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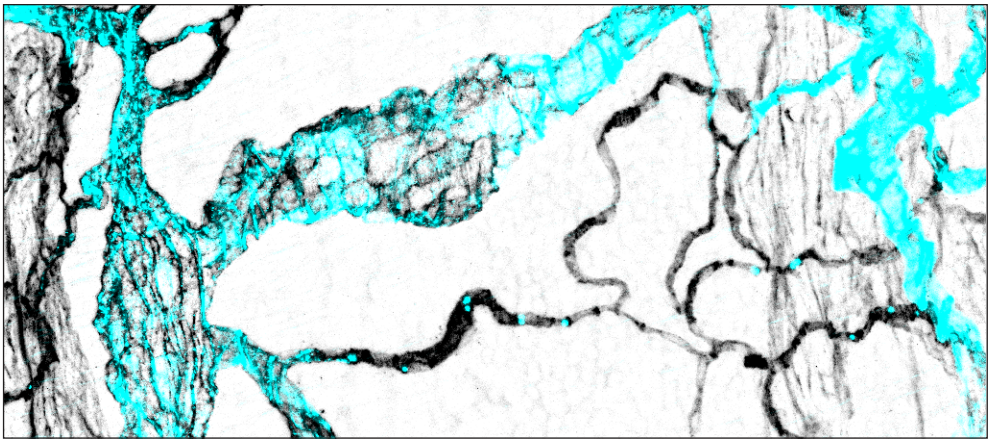
EMILIA KORHONEN

THE ENDOTHELIAL ANGIOPOIETIN-TIE SIGNALING PATHWAY IN DEVELOPMENT AND DISEASE



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THE ENDOTHELIAL ANGIOPOIETIN-TIE SIGNALING PATHWAY IN DEVELOPMENT AND DISEASE

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To my family

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* # Equal contribution

Publication III was included in the doctoral thesis of Anita Lampinen (2016).

ABBREVIATIONS

AAV	Adenovirus-associated virus
ABTAA	Ang2-binding and Tie2-activating antibody
Ad	Adenovirus
AJ	Adherens junction
AMD	Age-related macular degeneration
Ang	Angiopoietin
ARDS	Acute respiratory distress syndrome
BBB	Blood brain barrier
BEC	Blood endothelial cell
BM	Basement membrane
CNS	Central nervous system
COMP	Cartilage oligomeric matrix protein
Dll4	Delta like ligand 4
DP	Diabetic retinopathy
dpi	Days post-immunization
E	Embryonic day
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
FLIM	Fluorescence lifetime imaging microscopy
FN	Fibronectin
FOXO1	Forkhead box protein O1
FRET	Fluorescence resonance energy transfer
HIF	Hypoxia-inducible factor
HUVEC	Human umbilical vein endothelial cell
ICAM1	Intercellular adhesion molecule 1
Ig	Immunoglobulin
IL-1β	Interleukin-1β
LEC	Lymphatic endothelial cell
LECT2	Leukocyte cell-derived chemotaxin 2
LPS	Lipopolysaccharide
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NCID	Intracellular domain of Notch
P	Postnatal day
PCG	Primary congenital glaucoma
PlGF	Placenta growth factor
PLLV	Primordial longitudinal lymphatic vessel
pTD	Primordial thoracic duct
SC	Spinal cord
scRNA-seq	Single-cell RNA-sequencing
SMC	Smooth muscle cell
TEM	Tie2 expressing macrophage
Tie	Tyrosine kinase with Ig-like and EGF-like domains
TJ	Tight junction
TNF-α	Tumor necrosis factor alpha
VCAM1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin (Cadherin-5, Cdh5)
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VM	Venous malformation

ABSTRACT

The vascular system consists of hierarchical networks of blood vessels and lymphatic vessels. The main function of the blood vasculature is to transport oxygen and nutrients to tissues, whereas the unidirectional lymphatic network is required mainly for maintenance of tissue fluid homeostasis and lipid absorption. Growth of new blood and lymphatic vessels, called angiogenesis and lymphangiogenesis, is required during embryonic development and in physiological processes in adults. Malfunction of the vascular systems is associated with various diseases such as cancer, inflammation and neovascular eye diseases.

The functions of the vascular system are controlled by endothelial cells (ECs) lining the blood and lymphatic vessels. ECs are regulated by many growth factors including the endothelial angiopoietin (Ang) growth factor -Tie receptor signaling system. The two transmembrane tyrosine kinase receptors Tie1 and Tie2 form a receptor complex for angiopoietin growth factors, which regulate angiogenesis and lymphangiogenesis. Ang1 and Ang2 act as agonistic and context-dependent agonistic/antagonistic ligands of Tie2, respectively. However, Tie1 does not bind to angiopoietins. Instead, Tie1 participates in Ang-Tie2 signaling by forming a heterocomplex with Tie2.

Ang-Tie signaling is important in vascular development and it controls pathological angiogenesis as well as vascular remodeling and integrity in inflammatory conditions. For example, increased Ang2 concentrations predict poor patient survival in severe diseases. Overall, the Ang-Tie pathway can be considered to be a potential therapeutic target. However, little is known about the function of Tie1 and the regulation of the context-dependency of Ang2. In this thesis, we investigated the function of Tie1 in embryonic lymphatic vascular development, postnatal sprouting angiogenesis and pathological angiogenesis. We also aimed to understand how Tie1 regulates angiopoietin signaling and the function of angiopoietins in inflammation. Furthermore, we studied the function of Ang2 in neuroinflammation.

Constitutive Tie1-deletion results in embryonic lethality due to hemorrhage, edema and disruption of the microvasculature. Edema observed in Tie1-deleted embryos led us to hypothesize that Tie1 contributes to lymphatic vessel development. Indeed, we found that Tie1 was expressed in earliest lymphatic structures in developing embryos, called lymph sacs. The lymph sacs were fragmented in Tie1-deleted embryos. Analysis of different timepoints during development of Tie1-deleted embryos revealed that the edema and malformed lymph sacs precede the appearance of hemorrhages in these embryos. These results indicate that Tie1 is essential for normal lymphatic vessel development and that the lymphatic vessels are more sensitive to the loss of Tie1 than the blood vessels.

We next investigated the function of Tie1 in tumor angiogenesis. EC-specific deletion of Tie1 reduced tumor angiogenesis and growth due to increased EC apoptosis. Tie1 deletion also reduced postnatal sprouting angiogenesis in the retina through activation of the Notch pathway. Importantly, Tie1 deletion did not affect healthy vasculature in adult mice. Tie1 deletion inhibited tumor growth as effectively as blockers targeting the vascular endothelial growth factor receptor (VEGFR) system, which are currently used in the clinic. However, no additive effects were observed when these two treatment strategies were combined, whereas additive inhibition of tumor growth was observed when Tie1 deletion was combined with angiopoietin inhibition.

Combinatorial targeting of Tie1 and Ang2 also reduced angiogenesis in the retina, suggesting that targeting Tie1 in combination with Ang2 can improve anti-angiogenic therapy.

We next analyzed the role of Tie1 in angiopoietin signaling and function in inflammation. We found that angiopoietin stimulation promoted Tie1-Tie2 interaction and Tie1 was required for normal Tie2 trafficking. Tie1 deletion in mice reduced Ang1- and Ang2-induced vascular remodeling. Furthermore, Tie1-deficiency reduced Ang1-induced activation of Tie2 and downstream signaling and inhibited the Tie2 agonistic activity of Ang2. In lipopolysaccharide (LPS)-induced inflammation, the extracellular domain of Tie1 was rapidly cleaved and this was associated with reduced Tie2 activation and loss of Ang2 agonistic activity. Our results thus indicated that Tie1 is an important regulator of Ang-Tie2 signaling and agonistic function of Ang2 is decreased in inflammation.

Finally, we investigated the function of Ang2 in neuroinflammation. We found that Ang2 was induced in experimental autoimmune encephalomyelitis (EAE), a rodent model of multiple sclerosis (MS). Ang2 overexpression exacerbated the severity of EAE whereas its blockade ameliorated EAE. Similarly, treatment of the mice with ABTAA (Ang2-binding and Tie2-activating antibody), increased Tie2 phosphorylation and this reduced EAE severity, indicating that Tie2 activation is beneficial in this model. Our study showed that in neuroinflammation, Ang2 regulates the expression of EC adhesion molecules, blood brain barrier (BBB) integrity and recruitment of leukocytes into the central nervous system (CNS), as well as the pro-inflammatory polarization of CNS myeloid cells. These data implicate a role for Ang2 in autoimmune neuroinflammation.

Overall, this thesis provides insight into the function of Tie1 and Ang2. Our studies reveal previously unknown roles of Tie1 in lymphatic vessel development, postnatal sprouting angiogenesis as well as in pathological tumor angiogenesis. We found that Tie1 is a critical component of the angiopoietin-Tie2 signaling and in inflammation its cleavage is associated with reduced agonistic function of Ang2. Furthermore, our work provides novel possibilities for therapeutic targeting of Ang2 in neuroinflammation. A deeper understanding of the functions of Tie1 and Ang2 in disease pathogenesis should aid in the development of therapies targeting the Ang-Tie signaling system.

REVIEW OF THE LITERATURE

1. STRUCTURE AND FUNCTION OF THE VASCULAR SYSTEM

1.1 The blood vascular system

The blood vascular system is required for the transport of oxygen and nutrients to tissues and for the removal of carbon dioxide and metabolic waste products. All vertebrates have a closed circulatory system that consists of the systemic and the pulmonary circulation. Pumping of the heart circulates blood to the systemic and pulmonary circulations through a hierarchical network of blood vessels. All tissues except cartilage, lens, and the cornea are vascularized. Blood vessels are lined and regulated by ECs. The blood vasculature is organized into arteries, arterioles, capillaries, venules, and veins. Arteries carry oxygenated blood from the heart to the body, and veins carry blood from the body back to the heart. Capillaries between arteries and veins are mainly responsible for the exchange of oxygen and other nutrients as well as waste products between the blood and the interstitial fluid.

1.1.1 Blood vessel structure and function

Different types of blood vessels share similar structural features, but they vary according to their size and function. Large vessels, such as arteries and veins, have multiple layers of cellular and extracellular material, providing support and contractility. Large vessel walls are composed of three layers: an interior layer composed of endothelium and basement membrane (BM) (tunica intima), an intermediate layer composed of smooth muscle cells (SMCs), elastin, proteoglycans and nerves (tunica media); and an external layer (tunica externa), which consists of connective tissue with collagenous and elastic fibers. Small capillaries consist of a single endothelial layer surrounded by a BM and loose coverage of pericytes. Vessels with an increasing diameter such as arterioles and venules become covered with SMCs. Arteries have strong elastic walls to withstand the high blood pressure downstream from the heart, whereas veins have valves to prevent backflow of the blood. The valves are composed of thickened endothelium and connective tissue that form two luminal leaflets.

The basolateral side of the ECs is lined with the BM, which is a sheet-like structure that provides structural and mechanical support to the ECs and separates them from the underlying connective tissue. It is composed of mainly type IV collagen, laminin, heparan-sulfate proteoglycans, and nidogen/entactin. However, the molecular composition of BM varies in different tissues. Components of the vascular BM are needed for the initiation and resolution of angiogenesis, and they are regulated by matrix metalloproteinases and vascular integrins (Kalluri, 2003).

The primary function of the vasculature is to supply efficient oxygenation to tissues and return the deoxygenated blood to the lungs. Other homeostatic functions of the vasculature include hemostasis, lipid transport, and immune surveillance. Blood vessel functions are regulated by ECs. Besides acting as a barrier between blood and the tissues, ECs are essential for the maintenance of vascular homeostasis. ECs also have several other functions including regulation of vessel growth and remodeling, immune responses, cell adhesion, tissue metabolism and vascular permeability.

1.1.1.1 Regulation of vascular permeability

ECs control the passage of solutes and blood proteins into the tissues. They have different morphologies and functions in different type of vessels and organs. Exchange of gases and other substances occurs in the capillaries, which are divided into three major types: continuous, fenestrated, and sinusoidal. Continuous capillaries have complete endothelial lining and do not have openings in their walls. They are found in almost all vascularized tissues such as heart, lung, skeletal muscle, and brain. Fenestrated capillaries have small pores in their EC lining with a permeable diaphragm, which allows the transfer of macromolecules. Fenestrated capillaries are found in the small intestine, kidneys, and many endocrine organs. Sinusoidal endothelium has large fenestrations and a discontinuous basement membrane, which allows the passage of large solutes such as plasma proteins. Sinusoids are found in the liver, spleen, bone marrow, lymph nodes, and several endocrine glands (Aird, 2007; Augustin and Koh, 2017).

EC permeability is mediated by two pathways. In the paracellular pathway, the solutes and cells pass between the ECs, while in the transcellular pathway, they pass through the ECs. Transcellular permeability requires specialized membrane structures, such as vesicular vacuolar organelles (Dvorak and Feng, 2001). The paracellular route relies on the coordinated opening and closure of endothelial cell-cell junctions. EC junctions control vascular homeostasis by regulating the permeability to plasma solutes and leukocyte extravasation (Vestweber et al., 2009). Within EC junctions, ECs adhere to one another by adhesive transmembrane junctional proteins. Those form junctional structures that are linked to the intracellular actin cytoskeleton through intracellular partners, which stabilize the junctions.

ECs have two major type of junctions: adherens junctions (AJs) and tight junctions (TJs). The organization of the junctions depends on the functional needs of the perfused organ. AJs initiate cell-to-cell contacts and regulate EC growth and paracellular permeability. TJs provide a "barrier" by regulating permeability and maintaining cell polarity. TJs are highly abundant in the brain endothelium, where permeability needs to be tightly controlled. In turn, TJs in the postcapillary venules are poorly organized since dynamic trafficking of cells and proteins is required. Major components of the AJs are members of the cadherin family, whilst TJs are formed by members of the claudins as well as occludin and JAMs. VE-cadherin is the most prominent AJ protein, and it plays essential roles in regulating vascular permeability (Dejana, 2004; Dejana et al., 2009).

1.2 The lymphatic vascular system

Unlike the blood vascular system, the lymphatic system is a unidirectional network. It consists of blind-ended capillaries that drain fluid into pre-collectors and collecting vessels present in most organs. Some organs, including bone, adipose tissue, muscle, endocrine organs, as well as the parenchyma of the brain, liver, and kidney, lack or have a very sparse lymphatic network (Petrova and Koh, 2018). Recent research shows the presence of lymphatic vessels also in the eye and central nervous system, which were previously considered to lack lymphatic vasculature (Aspelund et al., 2014, 2015; Louveau et al., 2015; Park et al., 2014). The lymphatic vasculature maintains tissue fluid homeostasis by returning the fluid and solutes extravasated from the blood capillaries to the cardiovascular system. Lymph flows through lymph nodes into the thoracic duct, from where it is returned to the venous circulation at junctions with the subclavian vein.

1.2.1 Lymphatic vessel structure and function

The structure of lymphatic vessels varies depending on their function. Capillaries consist of a layer of partially overlapping lymphatic endothelial cells (LECs), which have discontinuous or no BM, and lack SMC coverage. Capillaries have oak-leaf shaped LECs with specialized, discontinuous "button-like" junctions, which serve as sites of fluid and immune cell entry. LECs are anchored to the extracellular matrix (ECM) by anchoring filaments that act as mechanosensors. These mechanosensors respond to increased pressure and permit fluid entry into the lymphatic vessels. From the initial lymphatics, lymph is transported to collector lymphatic vessels. These harbors both continuous "zipper-like" junctions to prevent leakage and intraluminal valves to prevent the backflow of the lymph. Collectors also have BM and SMC coverage. Lymph is transported by forces provided by the contraction of SMCs and skeletal muscle, respiratory movement, and the pressure pulse from adjacent arteries.

Lymphatic vessels drain fluid from the interstitial space of tissues and subsequently return it back to blood circulation via the thoracic duct. In addition to lymph transport, the lymphatic system has essential functions in immune surveillance and the uptake of dietary lipids. The immune system continually scans for extrinsic pathogens and mutated host cells. Lymphatic vessels take up pathogens together with the interstitial fluid and transport immune cells and free antigens to regional lymph nodes. In lymph nodes, antigen-presenting cells, T-cells, and B-cells act in concert to mount an immune response, and lymphatic vessels transport activated immune cells and antibodies to the circulation.

In addition to the transport of interstitial fluid and immune cells, lymphatic vessels are crucial in uptake and transport of dietary lipids. Uptake of nutrients from the gastrointestinal tract takes place in the intestinal villi. The majority of nutrients are transported by blood vessels, but high molecular weight lipids are taken up by the lymphatics due to the highly permeable structure of the intestinal lymphatic vessels, which are called lacteals.

2. BLOOD AND LYMPHATIC VESSEL DEVELOPMENT AND GROWTH

2.1 Embryonic development

Vascular network formation is a complex process, which is essential for vertebrate development. During mammalian embryonic development, blood vessels and the heart are the first organs to develop and function. Lymphatic vessels develop after the establishment of the blood vessel network and blood circulation. Many of the processes involved in embryonic vascular development also occur in adults during neovascularization, both during physiological and pathological conditions.

2.1.1 Vasculogenesis and angiogenesis

During embryonic development, the vasculature develops via vasculogenesis and angiogenesis. Vasculogenesis refers to the *de novo* formation of blood vessels from the mesoderm, whilst angiogenesis refers to the generation of new blood vessels from pre-existing vessels.

Vasculogenesis is driven by the differentiation of mesodermal cells to the endothelial lineage and their assembly to vessel structures.

In vasculogenesis, mesodermal stem cells produce hematopoietic cells and angioblasts. These cell types are both positive for vascular endothelial growth factor-2 (VEGFR2), the primary receptor for the VEGF growth factor (Ema and Rossant, 2003). These cells derive from the posterior primitive streak and migrate into embryonic and extraembryonic structures. In the yolk sac, they aggregate into blood islands that generate a primary capillary plexus. In addition, intraembryonic angioblasts assemble to form the cardinal vein and dorsal aorta. Together these structures form the vascular network. The primitive vascular network is pruned to form blood vessels by multiple mechanisms including sprouting angiogenesis, elongation, intussusception and, possibly, incorporation of circulating EC progenitors. Angiogenesis, the process of generation of new vessels from pre-existing blood vessels, is driven by EC proliferation. VEGF/VEGFR2 pathway is the most important signaling system in developmental angiogenesis. Indeed, mouse embryos lacking one allele of VEGF die at embryonic day (E) 9.5 (Carmeliet et al., 1996; Ferrara et al., 1996) and mice lacking VEGFR2 do not develop blood islands and die between E8.5-E9.5 (Shalaby et al., 1995).

The initial blood vessels are further remodeled and stabilized into the hierarchical vascular network consisting of arteries, veins and capillaries. Arterial-venous specification is determined by angioblast expression of arterial Ephrin-B2 or venous EphB4, specified by Notch signaling (Zhong et al., 2001). Vessel remodeling is induced by blood flow and changes in metabolic demands (Chung and Ferrara, 2011; Culver and Dickinson, 2010). Vessel maturation is associated with deposition of extracellular matrix (ECM) proteins and they are stabilized by recruitment of pericytes and SMCs induced by PDGF-BB (Chung and Ferrara, 2011; Hellström et al., 2007).

2.1.2 Lymphangiogenesis and lymphvasculogenesis

In mice, the majority of LECs arise from a subpopulation of ECs on the dorsal side of the cardinal vein. These cells start to express the transcription factor PROX1, which is induced by the SOX18 transcription factor at E9.5 (François et al., 2008). Together with the transcription factor COUP-TFII, PROX1 promotes LEC identity by downregulation of blood endothelial cell (BEC)-specific genes and upregulation of LEC-specific genes, such as VEGFR3, the receptor for the most crucial lymphatic growth factor VEGFC (Joukov et al., 1996; Lee et al., 2009). PROX1-positive cells start to proliferate and migrate from the veins as strings of cells, upstream of a VEGFC gradient. These cells fuse to form lymph sacs, composed of the primordial thoracic duct (pTD) and the primordial longitudinal lymphatic vessel (PLLV) (Hägerling et al., 2013) (**Figure 1**). The pTD and the cardinal vein share a connection for the return of the lymph into the blood circulation. Blood-lymph separation is maintained by platelet aggregation promoted by an interaction between platelet receptor CLEC2 and podoplanin expressed on LECs (Osada et al., 2012).

After the initial formation of “lymph sacs”, the lymphatic network expands from pre-existing lymphatic vessels in a process termed lymphangiogenesis. However, some vessels instead have a non-venous origin. In lymphvasculogenesis, blood-forming hemogenic ECs trans-differentiate into LECs and incorporate into the growing lymphatic network. Lymphatic vessels in the mesentery are formed from both embryonic veins and LEC clusters originating from hemogenic endothelium (Stanczuk et al., 2015). Furthermore, most lymphatic vessels in the lumbar dermis

(Martinez-Corral et al., 2015) and a proportion of lymphatic vessels in the heart arise from Tie2-negative non-endothelial origin (Klotz et al., 2015). However, a recent study suggests that LEC clusters in the lumbar skin originate from the developing blood vascular capillary plexus (Pichol-Thievend et al., 2018). The lymphatic capillary network is further remodeled, and the lymphatic vessels mature by development of valves and acquisition of SMCs and BM around the collectors, while the capillary junctions remodel from zippers to buttons (Yao et al., 2012). Maturation occurs in late gestation and continues during the early postnatal period. Lymphatic vasculature in different organs varies greatly depending on functional demands and physical restriction, which might reflect the different origins of lymphatic vessels (Petrova and Koh, 2018).

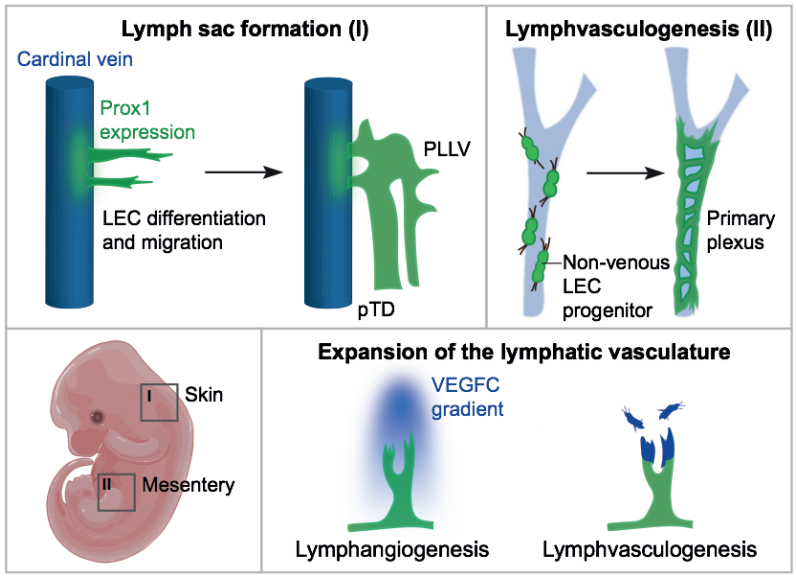

Figure 1. Development of the lymphatic vasculature. A major part of the lymphatic vasculature, for example in the skin cervical and thoracic regions, is formed from the lymph sacs composed of the primordial thoracic duct (pTD) and the peripheral longitudinal lymphatic vessel (PLLV). Some lymphatic vascular beds, for example in the mesentery, are formed via lymphvasculogenesis by assembly from non-venous-derived LEC progenitors. Further expansion of the lymphatic vasculature occurs by both lymphangiogenesis and lymphvasculogenesis. Adapted from (Karaman et al., 2017; Potente and Mäkinen, 2017). Embryo image from Biorender.

2.2 Postnatal development

The architecture of the vasculature is formed during embryonic development, although its growth and remodeling continues during early postnatal period. However, in some organs, the blood and lymphatic vasculatures develop postnatally. Physiological angiogenesis and lymphangiogenesis also occurs in adults.

2.2.1 Retinal angiogenesis

In mice, the retinal vasculature develops after birth. Thus, it is a widely used model for the study of angiogenesis. The retinal vasculature starts to grow around the optic nerve at the center of the retina and growth proceeds towards the peripheral retina, which allows for convenient identification of the growing vascular front (Fruttiger, 2002) (**Figure 2**). In sprouting angiogenesis, the ECs adopt different phenotypes that contribute to the formation of the new vessel network.

Indeed, tip cells at the front of the growing vasculature respond to VEGF by initial migration, whilst stalk cells behind the growing tip proliferate. Tip cells respond to a gradient of VEGF, and the proliferation of stalk cells depends on VEGF concentration. Both of these processes are mediated by VEGFR2-receptors in ECs (Gerhardt et al., 2003).

The retinal vasculature develops in close contact with an astrocyte scaffold that guides the extension of the tip cell filopodia by producing VEGF (Gerhardt et al., 2003). Notch signaling is also crucial for the determination of tip and stalk cell phenotypes. Tip cells have increased expression of Notch ligands such as Delta like ligand 4 (Dll4), which activates Notch signaling in the neighboring stalk cells. This in turn leads to expression of Notch target genes and suppression of the tip cell phenotype, restraining excessive tip cell migration (Hellström et al., 2007; Leslie et al., 2007; Siekmann and Lawson, 2007). The stalk cell phenotype is partly due to reduced VEGFR2 expression and increased VEGFR1 expression caused by Notch activation (Hellström et al., 2007). The tip cell phenotype is plastic, and cells continuously compete for the tip cell position (Jakobsson et al., 2010). Eventually, tip cells fuse and form loop structures, which have a lumen formed by stalk cells (Gerhardt et al., 2003; Herwig et al., 2011).

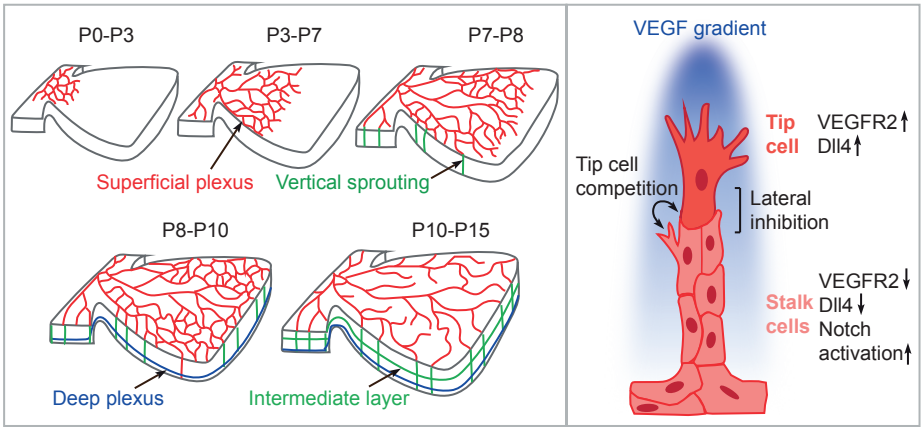

Figure 2. Development of the retinal vasculature. In the retina, blood vessel sprouting starts from the optic nerve at the center of the retina towards the periphery to cover the entire retina at around P8. This is followed by remodeling and maturation of the superficial plexus. Vertical sprouts are formed at about P7, followed by the formation of a deep plexus and intermediate plexus. Tip cells migrate towards the VEGF gradient and express high levels of VEGFR2 and Dll4, which activates Notch signaling in the neighboring cells to induce a stalk cell phenotype. Adapted from (Milde et al., 2013; Potente and Mäkinen, 2017)

2.2.2 Postnatal lymphatic vessel development

In most organs, lymphatic vessels are formed in the embryo and their maturation continues after birth. For example, remodeling and maturation of the cardiac lymphatic vessels continues postnatally until 2-3 weeks after birth in mice (Juszyński et al., 2008; Klotz et al., 2015; Mäkinen et al., 2001). However, the lymphatic vessels of the meninges and the Schlemm's canal are formed postnatally (Antila et al., 2017; Aspelund et al., 2014; Izen et al., 2018; Park et al., 2014). Lymphatic vessels in the meninges are completed during the first postnatal month and similarly to lymphatic vessels in the intestine, their development requires VEGFC/VEGFR3 signaling (Antila et al., 2017; Nurmi et al., 2015). Schlemm's canal is considered a hybrid vessel with both blood and lymphatic-like features since it does not express all of the common lymphatic markers.

Primordial Schlemm's canal is formed by blood ECs budding from the choroidal vein at birth. In contrast to the lymphatic vessel development initiating from the cardinal vein, in the developing Schlemm's canal, PROX1 expression starts at P4, only after the initial formation of the primitive Schlemm's canal (Aspelund et al., 2014; Park et al., 2014). In addition to the VEGFC/VEGFR3 pathway, also Ang1/Ang2 and Tie2 are crucial for Schlemm's canal development (Aspelund et al., 2014; Kim et al., 2017; Thomson et al., 2014).

2.2.3 Angiogenesis and lymphangiogenesis in the adult

Vessels in most adult tissues are quiescent. EC proliferation is high in embryos, but low in adults (Risau, 1997). Physiological angiogenesis occurs in the adult tissues only during pregnancy, menstrual cycle, wound healing, skeletal muscle hypertrophy, and in fat mass expansion (Cao, 2007; Gustafsson, 2011; Tonnesen et al., 2000; Zygmunt et al., 2003). However, the main angiogenic growth factor VEGF is also required for the homeostasis of adult vasculature (Lee et al., 2007). Similar to angiogenesis, little physiological lymphangiogenesis occurs in the adults except for the development of the corpus luteum, ovarian follicle growth, and during wound healing (Alitalo et al., 2005; Otsuki et al., 1986; Rutkowski et al., 2013). In adults, VEGFC is required for the maintenance of lymphatic vessels in the intestine and meninges (Antila et al., 2017; Nurmi et al., 2015), whereas Ang-Tie2 signaling is required for the maintenance of the Schlemm's canal (Kim et al., 2017). In addition to physiological conditions, angiogenesis and lymphangiogenesis occur in several pathological conditions, such as tissue inflammation and cancer.

3. MOLECULAR REGULATORS OF THE VASCULAR SYSTEM

3.1 VEGF-VEGFR signaling pathway

VEGFs and their receptors constitute the main EC signaling pathway that regulates angiogenesis and lymphangiogenesis. The VEGF-VEGFR family consists of VEGF, VEGFB, VEGFC, VEGFD, and placenta growth factor (PlGF) (Leung et al., 1989; Maglione et al., 1991; Olofsson et al., 1996; Park et al., 1994), and their receptors VEGFR1, VEGFR2, and VEGFR3 (Matthews et al., 1991; Pajusola et al., 1992; Shibuya et al., 1990; Terman et al., 1991). VEGFs have distinct receptor-binding patterns and they signal via VEGFR homodimers or heterodimers. VEGF is the primary angiogenic ligand that signals predominantly via VEGFR2 (Karaman et al., 2018). VEGFR1 binds VEGF, VEGFB, and PlGF. VEGFR1 functions mainly as a negative regulator of VEGF signaling, as it has weaker kinase activity than VEGFR2, but has higher affinity for VEGF than VEGFR2 (Ho et al., 2012; Shibuya and Claesson-Welsh, 2006). VEGFC and VEGFD induce VEGFR3 activation for lymphangiogenesis (Alitalo et al., 2005). VEGF-VEGFR signaling is also modulated by neuropilin co-receptors and other molecules such as VE-cadherin, integrins, and Ephrin-B2 (Becker et al., 2005; Chen et al., 2010; Coon et al., 2015; Sawamiphak et al., 2010; Soker et al., 1998; Wang et al., 2010).

Following ligand binding, the VEGF receptors undergo homo- or heterodimerization, which leads to auto- or transactivation of receptor tyrosine residues. This results in recruitment and activation of downstream signaling molecules, which mediate activation of specific signaling pathways for regulation of EC migration, survival and proliferation (Koch and Claesson-Welsh, 2012).

3.2 Notch signaling pathway

The Notch pathway is an evolutionarily conserved signaling system that modulates cell fate decisions during many developmental processes (Artavanis-Tsakonas et al., 1999). In ECs, it is essential for arterial specification, sprouting angiogenesis, and vessel maturation. The Notch pathway regulates angiogenesis and tip/stalk cell specification together with the VEGF-VEGFR pathway (Thomas et al., 2013). Cell surface-expressed Notch receptors (Notch1-Notch4) bind membrane-bound ligands Jagged1, Jagged2 and Dll1, Dll3, and Dll4 on adjacent cells. This restricts the Notch pathway to regulation of signals in neighboring cells, termed juxtacrine signaling. Ligand binding to Notch receptor induces its proteolytic cleavage by the disintegrin and metalloproteases (ADAM) family proteins and γ-secretase. The Notch intracellular domain (NICD) is released from the plasma membrane, and it translocates to the nucleus where it forms a complex with RBPJ protein to induce the expression of Notch target genes (Gridley, 2010; Phng and Gerhardt, 2009). Genetic loss-of-function mouse models have revealed that Notch signaling is required for vascular development, and that it also plays a role in tumor angiogenesis (Kofler et al., 2011).

3.3 Ang-Tie signaling pathway

In addition to the VEGF-VEGFR signaling system, the Ang-Tie system is a second EC-specific signaling pathway necessary for blood and lymphatic vessel development. The Ang-Tie pathway is considered to function in vascular remodeling and maturation after the initial angiogenic actions of the VEGF-VEGFR pathway. In addition to its role in vascular development, the Ang-Tie pathway also regulates postnatal angiogenesis, vessel remodeling, and it maintains vascular homeostasis by regulating vascular permeability and tissue inflammation in adults (Augustin et al., 2009; Eklund et al., 2017).

3.3.1 Structure and expression of the Ang and Tie molecules

The Ang-Tie pathway consists of the receptor tyrosine kinases (RTKs) Tie1 and Tie2 and the angiopoietin growth factor ligands (**Figure 3**). Ang1 is an agonistic ligand of Tie2, whilst Ang2 is a context-dependent agonist/antagonist ligand for Tie2 (Daly et al., 2006; Davis et al., 1996; Maisonpierre et al., 1997; Yuan et al., 2009). Additionally, less characterized angiopoietins, Ang4 in humans and its mouse orthologue Ang3, exist (Valenzuela et al., 1999).

Angiopoietins are composed of a C-terminal fibrinogen-like domain, a central coiled-coil domain linked to it via a linker peptide and an N-terminal angiopoietin-specific superclustering domain. The fibrinogen-like domain is required for receptor binding, whereas the coiled-coil domain mediates dimerization of angiopoietins. The short N-terminal superclustering domain forms ring-like structures that further clusters dimers to higher oligomeric forms (Davis et al., 2003; Kim et al., 2005; Procopio et al., 1999). Ang1 is expressed by peri-endothelial SMCs and pericytes, and several other cell types such as retinal neurons and myocardium during heart development (Davis et al., 1996; Park et al., 2017; Suri et al., 1996). Thus, Ang1 activates endothelial Tie2 in a paracrine manner. In contrast, Ang2 is an autocrine ligand expressed by ECs, wherein it is stored in Weibel-Palade bodies (WBPs) (Fiedler et al., 2004). Ang2 expression and secretion from the WPBs is increased by several factors, including hypoxia, VEGF and inflammatory signals (Fiedler

et al., 2004; Oh et al., 1999). Recent work has shown that Ang4 is expressed in the astrocytes of the developing retina, suggesting it also functions in a paracrine manner similar to Ang1 (Elamaa et al., 2018).

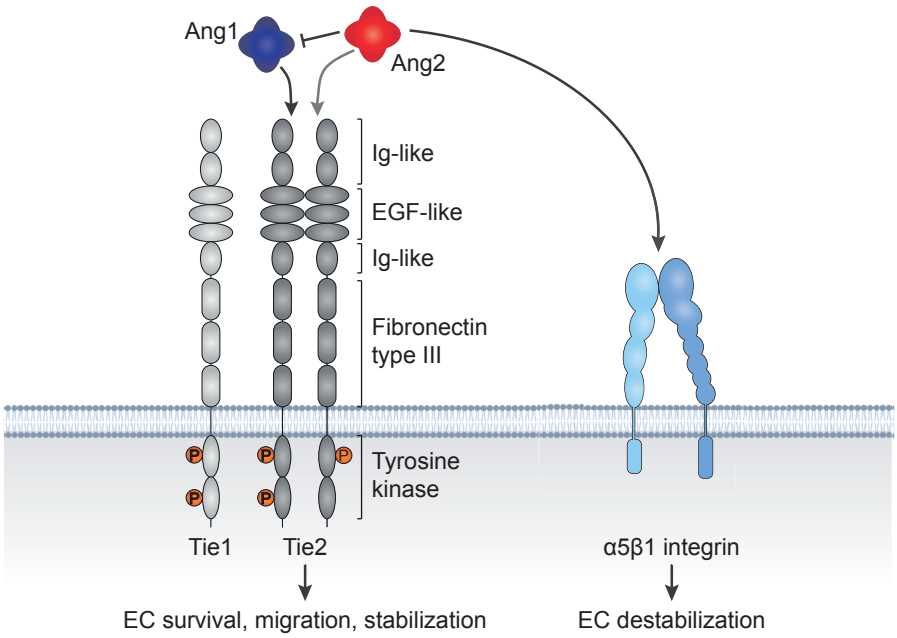

Figure 3. Structure and signaling interactions of the Ang-Tie pathway. Ang1 is a strong agonistic ligand, whereas Ang2 can act as a weak agonistic or antagonistic ligand of Tie2. Tie1 interacts with Tie2 in a heterocomplex. When Tie2 levels are low, Ang2 can also signal via α5β1-integrin. Ig-like, immunoglobulin-like domain; EGF-like, epidermal growth factor-like repeat; P, phosphorylation; EC, endothelial cell. Adapted from (Saharinen et al., 2017). Lipid bilayer image from Biorender.

The Tie receptors mediate angiopoietin growth factor signaling. These single transmembrane receptors have an extracellular domain responsible for ligand binding and a split intracellular tyrosine kinase domain that mediates activation of downstream signaling pathways. Tie1 and Tie2 have similar overall structures, and especially their intracellular domains are highly conserved (Schnürch and Risau, 1993). The extracellular domain consists of two immunoglobulin (Ig)-like domains, three epidermal growth factor (EGF)-like domains, a third Ig-like domain, and finally three fibronectin type III (FNIII) domains adjacent to a transmembrane domain. The intracellular domain consists of a tyrosine kinase domain (Barton et al., 2006; Macdonald et al., 2006). The ligand binding region contains the Ig domains and the two N-terminal EGF domains, which fold together to form a compact arrowhead-shaped structure (Barton et al., 2006; Macdonald et al., 2006). Ang1 and Ang2 bind to the same site on Tie2 with similar affinities (Fiedler et al., 2003). All angiopoietins bind to Tie2 but they do not bind to Tie1 (Davis et al., 1996; Lee et al., 2004; Maisonpierre et al., 1997). Historically, Tie1 has been considered an orphan receptor, with no known ligand. However, a recent study has suggested that leukocyte cell-derived chemotaxin 2 (LECT2) serves as a ligand for Tie1 (Xu et al., 2019).

The Tie receptors are expressed in blood and lymphatic ECs and are almost exclusively endothelial-specific. In addition, Tie1 is expressed in some hematopoietic cells such as platelets (Armstrong et al., 1993; Batard et al., 1996; Tsiamis et al., 2000), whereas Tie2 expression is

found in a subpopulation of type M2 monocytes, referred as Tie2-expressing monocytes (TEMs), in muscle satellite cells, hematopoietic stem cells in the bone marrow niche and pericytes (Abou-Khalil et al., 2009; De Palma et al., 2005; Shaw et al., 2004; Teichert et al., 2017). Tie2 expression is constant, whereas Tie1 expression is dynamically regulated. For example, the expression of Tie1 is increased in tumor vasculature, by disturbed blood flow in atherosclerosis-prone areas, and by hypoxia and VEGF stimulation (Kaipainen et al., 1994; McCarthy et al., 1998; Porat et al., 2004)

3.3.2 Physiological roles of the Ang-Tie system

3.3.2.1 Embryonic and postnatal blood vessel development

Tie2-deficient embryos die between E10.5-E12.5 due to defective development of the cardiovascular system (Dumont et al., 1994). This early lethality and severe cardiovascular defects are phenocopied in Ang1-deficient embryos (Suri et al., 1996). Tie2 and Ang1 are required for the development of heart endocardium and myocardial trabeculation. Indeed, the deleted mice exhibit only a few myocardial trabeculations and impaired endocardial-myocardial interactions (Dumont et al., 1994; Jeansson et al., 2011; Qu et al., 2019a; Sato et al., 1995; Suri et al., 1996). Myocardial-specific Ang1-deletion displays a similar phenotype as global Ang1 deletion, suggesting that the vascular defects are secondary to the cardiac defects (Jeansson et al., 2011). Tie2 is dispensable for the early cardiovascular and angiogenic development, but in Tie2-deficient embryos, the primary capillary plexus fails to remodel and mature. The plexus in Tie2- and Ang1-deficient embryos is less complex, poorly organized, and has fewer pericytes and SMCs (Patan, 1998; Sato et al., 1995; Suri et al., 1996). In addition, Tie2-deleted embryos exhibit impaired arteriovenous specification (Chu et al., 2016). Surprisingly, Ang1 deletion after E13.5 did not affect the vasculature indicating that it is dispensable for the maintenance of vascular integrity in homeostatic conditions (Jeansson et al., 2011).

In contrast to Ang1, Ang2 is not required for developmental angiogenesis, but interestingly, transgenic Ang2-overexpressing embryos exhibit a similar phenotype as Ang1-deleted mice. Ang2 overexpression impairs blood vessel formation and leads to embryonic lethality in mid-gestation. These observations have led to the concept that Ang2 acts as an antagonist of Ang1 and Tie2 (Maisonpierre et al., 1997).

Tie1-deleted embryos die starting at E13.5, depending on the genetic background, due to edema and hemorrhages (Patan, 1998; Puri et al., 1995). Although Tie1 deletion did not result in similar cardiac defects as in Tie2- and Ang1-deleted mice, Tie1 was shown essential for aortic valve development during late gestation (Qu et al., 2019b). Tie1 is not required for the early steps of vascular development, but the integrity of the microvasculature is lost in Tie1-deficient embryos (Puri et al., 1995). Indeed, even the loss of both Tie receptors does not interfere with early vascular development. However, experiments using double mutant chimeric mice revealed that both receptors are required during late organogenesis and in the mature adult vasculature (Puri et al., 1999).

The Ang-Tie pathway is also crucial for postnatal angiogenesis. Neonatal deletions of Tie2, Ang1, and Ang2 result in reduced retinal angiogenesis in mice (Gale et al., 2002; Lee et al., 2013; Park et al., 2017; Savant et al., 2015). Although Ang2 is dispensable for the developmental angiogenesis in embryos, its deletion impairs postnatal vascular remodeling in the retina and inhibits the regression of hyaloid vessels in the vitreous of the eye (Gale et al., 2002). Ang1 is also required

for the formation of the retinal vasculature in postnatal mice. In the retina, Ang1 promotes vascular network formation via Tie2 activation in the vasculature and via αvβ5-integrin in the retinal astrocytes. In the astrocytes, Ang1-induced activation of αvβ5-integrin promotes fibronectin deposition, which guides directional angiogenesis (Lee et al., 2013). Furthermore, astrocyte-expressed Ang4 is required for venous remodeling in the retina (Elamaa et al., 2018).We and others have shown that also Tie1 is required for the retinal angiogenesis, and its EC-specific deletion reduces the migration of the vascular front, number of branch points, filopodia, and sprouts (Study II, D'Amico et al., 2014; Savant et al., 2015). In tip cells, Tie1 inhibits Tie2 cell surface presentation, whereas in stalk cells, Tie1 sustains Tie2 activation (Savant et al., 2015). Double deletion of both receptors suppressed sprouting and induced vessel regression (Savant et al., 2015). Tie2 deletion also induces leakage of the retinal vessels. However, Tie2 is not necessary for the maintenance of the retinal vessels in adults (Park et al., 2017).

3.3.2.2 Embryonic and postnatal lymphatic vessel development

Although Ang2 is dispensable for embryonic blood vessel development, the Ang2-deleted mice have severe lymphatic defects postnatally. Ang2-deleted mice in the 129/J genetic background are born at normal Mendelian ratios, but die two weeks after birth due to severe chylous ascites, whereas Ang2 deletion in C57bl/6J mice results in transient postnatal chylous ascites due to defective lymphatic vessel development (Fiedler et al., 2003; Gale et al., 2002). Ang2-deleted mice display defective lymphatic valve development and lymphatic collector maturation as well as increased SMC recruitment to lymphatic capillaries (Dellinger et al., 2008; Gale et al., 2002; Shen et al., 2014a). However, Ang2 deletion does not affect the early development of lymph sacs in the embryo (Dellinger et al., 2008). The importance of Ang2 in lymphatic development and maturation was also shown by the administration of Ang2 blocking antibodies, which induced swelling and hemorrhages in embryos. Ang2 inhibition resulted in reduced LEC proliferation and sprouting, whereas Ang2 overexpression increased the proliferation of LECs. Ang2 was also found to regulate lymphatic junctions. Ang2 inhibition disrupted the zipper junctions in lymphatic collectors, and zippers failed to mature to buttons in the initial lymphatics (Zheng et al., 2014). Interestingly, expression of Ang1 from the Ang2 genetic locus rescued the lymphatic defects in Ang2-deleted mice, suggesting that in contrast to embryonic blood vessel development, Ang2 can function as a Tie2 agonist in the lymphatic vessels (Dellinger et al., 2008; Gale et al., 2002).

In addition to the blood vasculature, the Tie receptors are also crucial for lymphatic vessel development. We and others have shown that the edema observed in Tie1-deleted embryos is due to defective lymphatic vessel development. Tie1 deletion resulted in disrupted and disorganized lymph sac formation (Study I-II, D'Amico et al., 2010, 2014; Qu et al., 2010). Similar to Ang2 deletion, Tie1 was also later shown to be necessary for lymphatic valve development and maturation of lymphatic collecting vessels in embryos and postnatally. Additionally, Tie1-deletion was shown to result in abnormal SMC recruitment in initial lymphatics and reduce lymphatic drainage in the intestine and the ear skin (Qu et al., 2015; Shen et al., 2014a). Studies using inducible Tie2-deleted mice, in which Tie2 deletion was induced at E12.5, enabled the analysis of embryonic lymphatic development. Conditional Tie2-deletion resulted in edema, and only a few lymphatic vessels were observed in embryos with lymphatic-specific Tie2 deletion (Souma et al., 2018). In contrast, Tie2 has been shown to be dispensable for postnatal lymphatic vessel development (Shen et al., 2014a).

The function of the Ang-Tie system has also been studied in the lymphatic-like vessels of the eye, the Schlemm's canal, which develops postnatally. The deletion of Tie2 or Ang1/Ang2 resulted in impaired development of the Schlemm's canal leading to glaucoma (Kim et al., 2017; Thomson et al., 2014). Ang1 was shown to be the primary Tie2 ligand in the Schlemm's canal, although double deletion of Ang1/Ang2 resulted in a more severe phenotype than Ang1 deletion alone (Thomson et al., 2017). In line with this, double deletion of Ang1 and Ang2 in embryos, but not single deletion of either ligand alone, resulted in edema, resembling the swelling observed in the Tie2-deleted embryos (Souma et al., 2018). Furthermore, the deletion of Tie2 or both Ang1 and Ang2 inhibited the formation of ascending vasa recta in the kidney, which, similar to Schlemm's canal, is a lymphatic-like vessel (Kenig-Kozlovsky et al., 2018). Altogether, these results support the notion that Ang1 and Ang2 have overlapping functions in the lymphatic vasculature.

3.3.3 Signaling via the Ang-Tie system

The Ang-Tie system has a unique way of signaling that depends on the microenvironment and cellular compartment. In EC monolayers, angiopoietin stimulation translocates Tie receptors to cell-cell junctions, whereas in motile cells, angiopoietins induce the translocation of the Tie receptors to the trailing cell-ECM contacts (Fukuhara et al., 2008; Saharinen et al., 2008). The subcellular localization of the Tie receptors affects the activation of downstream signaling pathways and the subsequent EC responses, which could explain the different responses in vessels during quiescence and remodeling (Fukuhara et al., 2008; Saharinen et al., 2008). Ligand binding induces Tie2 clustering and activation, leading to recruitment of a variety of downstream signaling molecules.

3.3.3.1 Ang1-induced Tie2 activation

Ang1 is a potent Tie2 agonist. Acting via Tie2, Ang1 promotes EC survival, vessel stability, and EC barrier function. At cell-cell junctions, Ang1 induces the formation of Tie2-Tie2 trans-associated complexes (Fukuhara et al., 2008; Saharinen et al., 2008). Junctional Ang1-Tie2 complexes promote EC survival via the PI3K-Akt pathway, which activates survival-promoting pathways by increasing the exression of eNOS and survivin and suppressing Caspase9, a mediator of apoptosis (Babaei et al., 2003; DeBusk et al., 2004; Harfouche et al., 2002; Kim et al., 2000; Kontos et al., 1998; Papapetropoulos et al., 2000). Akt also phosphorylates Forkhead box protein O1 (FOXO1), which leads to its nuclear exclusion and the suppression of its target genes regulating cell growth and metabolism (Daly et al., 2004; Kim et al., 2000; Wilhelm et al., 2016). Ang1-Tie2 signaling also acts as an anti-inflammatory signal by recruitment of ABIN2, which inhibits the pro-inflammatory NF-kB signaling pathway (Hughes et al., 2003; Tadros et al., 2003).

Ang1-Tie2 activation also promotes vascular stability by regulating EC-EC junctions and the actin cytoskeleton (David et al., 2011; Gamble et al., 2000). Ang1-mediated Tie2 phosphorylation inhibits VEGF and inflammatory cytokine-induced loss of VE-cadherin by stabilizing VE-cadherin at EC-EC junctions (Gamble et al., 2000; Gavard et al., 2008). Tie2 activation leads to sequestration of Src, which mediates VEGF induced permeability by inducing VE-cadherin internalization (Gavard et al., 2008).

Furthermore, pericyte-expressed Ang1 activates Tie2 to promote a quiescent vascular phenotype via the recruitment of pericytes. In addition to the ECs, also pericytes express Tie2 (Cai et al., 2008; Iularo et al., 2003; Teichert et al., 2017), which can limit angiogenesis via calpain, Akt, and

FOXO3A. These results extend the current concept on endothelial Ang-Tie signaling and suggest a revised bi-directional, reciprocal model, which includes both EC- and pericyte expressed Tie2 (Teichert et al., 2017). In contrast to quiescent ECs, Ang1 activates partly different signaling pathways in motile cells. Matrix-anchored Ang1 induces Tie2 translocation to and in-cis association in the trailing cell-ECM contacts, thus promoting matrix adhesion and EC migration via the activation of Erk and DokR pathways (Fukuhara et al., 2008; Saharinen et al., 2008).

3.3.3.2 Context-dependency of Ang2

Ang2 is considered to be a context-dependent agonistic/antagonistic ligand for Tie2, and its ability to promote Tie2 activation depends on a variety of conditions (Saharinen et al., 2017). Ang2 can inhibit Ang1-Tie2 signaling as it binds Tie2 with similar affinity as Ang1, but induces a much weaker Tie2 activation (Daly et al., 2006; Fiedler et al., 2003; Maisonpierre et al., 1997). Thus, since both Ang1 and Ang2 compete for Tie2 binding, but Ang2 has a weaker Tie2 activating activity, Ang2 can be viewed as an antagonist in conditions where Ang2 levels are high compared to Ang1 (Maisonpierre et al., 1997; Tabruyn et al., 2010).

In inflammation, Ang1 promotes endothelial barrier integrity, whereas Ang2 destabilizes the quiescent vasculature by inhibiting Ang1-Tie2 signaling (Gamble et al., 2000; Scharpfenecker et al., 2005; Tabruyn et al., 2010). Ang2 expression is increased in several inflammatory conditions, in which Ang2 inhibits Ang1-Tie2 signaling, leading to increased nuclear localization of FOXO1, which further stimulates Ang2 transcription (Ghosh et al., 2015). In contrast, Ang2 functions as a Tie2 agonist in tumor vasculature and in stressed ECs, which have reduced Ang1-Tie2 signaling (Daly et al., 2006, 2013). Ang2 acts as a Tie2 agonist also in the lymphatic vasculature due to lack of tyrosine phosphatase VE-PTP in LECs (Souma et al., 2016). The absence of VE-PTP function in LECs lowered the threshold for Tie2 activation, thus allowing the weak agonist Ang2 to activate Tie2 in the LECs. In contrast, only Ang1 activated Tie2 in BECs, which expressed VE-PTP (Souma et al., 2018).

The context-dependency of Ang2 can depend on its multimerization. The crystal structure of Tie2 FNIII domains indicates that Tie2 dimerization in cis is mediated via FNIII domains. In the dimerized Tie2, the ligand-binding domains are far apart, facilitating Tie2 dimerization and activation by multimeric but not dimeric angiopoietins. Based on these results, the lower oligomerization state of Ang2 would explain its lower agonistic effects compared to Ang1, which exists mostly in high-order oligomers (Davis et al., 2003; Kim et al., 2005; Leppänen et al., 2017).

3.3.3.3 Tie1 signaling

Tie1 does not bind the angiopoietin growth factors, but it directly interacts with Tie2 in a heterocomplex (Saharinen et al., 2005). Angiopoietin stimulation translocates Tie1 to EC-EC contacts together with Tie2 and promotes its activation. Ang1-induced Tie1 activation is Tie2-dependent and much weaker than Tie2 activation (Saharinen et al., 2005, 2008; Savant et al., 2015). The function of Tie1 in Tie2 regulation is not completely understood. In vitro studies have indicated that Tie1 acts as a Tie2 inhibitor. Tie1 silencing or cleavage was shown to enhance Ang1- and Ang2-induced Tie2 activation (Marron et al., 2007; Seegar et al., 2010; Yuan et al., 2007). Furthermore, a computational model based on these studies suggested that Tie1 acts as a Tie2 inhibitor. However, this model illustrated the function of the Tie receptors in the tip cell population without considering the junctional localization of the receptors (Zhang et al., 2019). In contrast, our in vivo data indicates that Tie1 is a positive regulator of Tie2 signaling (Study II-III, D'Amico

et al., 2014; Korhonen et al., 2016). Interestingly, Tie1 was shown to regulate Tie2 signaling differently in the endothelial tip cells vs. the remodelling stalk cells (Savant et al., 2015). These results indicate that the function of Tie1 depends on the subpopulation of ECs.

The signaling pathways mediated by Tie1 are poorly understood. Ligand stimulation of CSF-1 receptor/Tie1 chimera was shown to result in receptor autophosphorylation and activation of PI3K and Akt, which promoted EC survival (Kontos et al., 2002). The ectodomain of Tie1 is proteolytically cleaved in response to shear stress and various other stimuli, such as VEGF and inflammatory cytokines (Chen-Konak et al., 2003; Marron et al., 2007; Singh et al., 2012; Yabkowitz et al., 1999). Following Tie1 cleavage, the Tie1 endodomain in the cytosol was shown to associate with the tyrosine phosphatase and adapter protein SHP2, suggesting that the endodomain has ligand-independent functions (Marron et al., 2000). Due to the responsiveness of Tie1 to shear stress, it has been suggested to be a component of the endothelial mechanosensory complex (Woo and Baldwin, 2011). Tie1 deletion in isolated murine aortic endothelial cells increased shear stress-mediated Tie2 and eNOS phosphorylation (Woo et al., 2011). Furthermore, Tie1 was also involved in regulating the response of lymphatic valve forming cells to turbulent flow (Qu et al., 2015), further supporting a role for Tie1 in mechanotransduction.

Lack of a known ligand for Tie1 has complicated the studies of Tie1-mediated signaling pathways. A recent study suggested LECT2 to be a functional ligand of Tie1 (Xu et al., 2019). LECT2 is a secretory protein, mainly expressed in hepatocytes and to a lesser degree in ECs. LECT2 binding to Tie1 was shown to interrupt Tie1/Tie2 heterodimerization and increase Tie2/Tie2 homodimerization, which resulted in reduced Tie1 activation and increased Tie2 activation. In the context of liver fibrosis, LECT2 binding to Tie1 was suggested to inhibit portal angiogenesis and promote sinusoid capillarization (Xu et al., 2019). Overall, these results strengthened the notion that Tie1 is involved in multiple processes and Tie1 signaling is context-dependent.

3.3.3.4 Ang-Tie interaction with integrins

Recent studies have shown that Ang-Tie signaling is modulated by the integrins, which are the most critical receptors in mediating cell adhesion to the ECM. They are heterodimeric receptors that consist of α and β subunits, which confer specificity to ECM ligands. Vascular cells express a variety of integrins that regulate the EC-ECM interplay in angiogenesis and inflammation (Pulous and Petrich, 2019). Several studies have shown that angiopoietins can bind to and activate integrins. Ang2 has been shown to interact with α5β1-integrin in Tie2-negative tumor cells (Imanishi et al., 2007; Lee et al., 2014a). Tumor ECs and angiogenic tip cells of the retina were shown to express low levels of Tie2, but high levels of Ang2 and integrins. In angiogenic cells, Ang2 induced integrin activation and EC migration in a Tie2-independent manner (Felcht et al., 2012; del Toro et al., 2010). Furthermore, Ang1 has been shown to signal via avβ5-integrin in retinal astrocytes to promote retinal angiogenesis (Lee et al., 2013).

Integrins have also been suggested to fine-tune Tie receptor signaling. Tie receptors were shown to form a complex with α5β1 and avβ3-integrins through their ectodomains in a fibronectin-dependent manner, and α5β1-integrin was required for Ang1-induced angiogenesis (Cascone et al., 2005; Dalton et al., 2016). The interaction between angiopoietins and integrins is weaker than angiopoietin binding to Tie2, indicating that Tie2 is the primary receptor for the angiopoietins (Felcht et al., 2012). However, low Tie2 expression is associated with angiogenic vessel sprouting and several inflammatory conditions in which Ang2 expression is upregulated (Felcht et al., 2012).

Interestingly, Ang2, but not Ang1, was shown to activate α5β1-integrin in EC monolayers, where Tie2 was silenced. Ang2-induced α5β1-integrin activation is mediated via the Ang2 N-terminus. Activation of α5β1-integrin led to the formation of stress fibers and destabilization of the EC monolayer (Hakanpaa et al., 2015). These data indicate that in conditions where Tie2 expression is low, Ang2 can signal via the integrins.

3.3.3.5 Regulation of Ang-Tie pathway by VE-PTP

VE-PTP is an EC-specific tyrosine phosphatase that is known to regulate Ang-Tie signaling (Fachinger et al., 1999). VE-PTP is essential during embryonic development, as VE-PTP-deleted embryos die between E9.5 and E11 due to abnormal angiogenesis (Bäumer et al., 2006; Dominguez et al., 2007). VE-PTP associates with Tie2 and negatively regulates its activation via dephosphorylation. In allantois explants and in juvenile mice, VE-PTP inhibition promoted Tie2 phosphorylation, EC proliferation, and vessel enlargement, suggesting that VE-PTP controls blood vascular development by balancing Tie2 activation. Additionally, VE-PTP regulated Tie2-dependent Tie1 activation (Winderlich et al., 2009).

VE-PTP also associates with VE-cadherin and enhances its adhesive function (Nawroth et al., 2002; Nottebaum et al., 2008). Interestingly, VE-PTP inhibition was shown to improve endothelial barrier function in response to LPS or VEGF in a Tie2-dependent manner, but independently of VE-cadherin (Frye et al., 2015). Mechanistically, Tie2 activation promoted activation of small GTP binding protein Rap1, which stabilized the cortical actin cytoskeleton via Rac1. However, in the absence of Tie2, VE-PTP inhibition increased vascular permeability via increased VE-cadherin internalization (Frye et al., 2015). In addition to Tie2 and VE-cadherin, VE-PTP has also been shown to dephosphorylate VEGFR2. VE-PTP was shown to regulate VEGFR2 phosphorylation via Tie2, suggesting that Tie2 promotes vascular stability partly via the downregulation of VEGFR2 activation through VE-PTP (Hayashi et al., 2013). VE-PTP has also been shown to regulate the agonistic vs. antagonistic functions of Ang2 by modulating Tie2 receptor sensitivity (Souma et al., 2018). These results indicate that Tie2 activation is tightly regulated by VE-PTP.

4. BLOOD AND LYMPHATIC VESSELS IN DISEASE

The blood and lymphatic vasculatures are involved in various diseases. Pathological angiogenesis and lymphangiogenesis share many common features with developmental angiogenesis and lymphangiogenesis. However, pathological angiogenesis and lymphanigiogenesis are less coordinated, leading to malformed and poorly functional vessels. Pathological angiogenesis and vascular leakage occur for example in cancer, neovascular eye diseases, and inflammation. Lymphatic dysfunction is associated with lymphedema, and tumor-induced lymphangiogenesis contributes to tumor metastasis.

4.1 Blood vasculature in disease

4.1.1 Tumor angiogenesis

When a tumor reaches circa 1-2 mm in diameter, hypoxia and poor delivery of nutrients triggers the requirement for angiogenesis (Folkman, 1971). Tumor progression from a benign to a malignant state involves an angiogenic switch, in which the balance between angiogenic factors

and angiogenesis inhibitors is disturbed (Folkman et al., 1989). Hypoxic conditions in the tumor promote the expression of hypoxia-inducible transcription factors (HIFs), which induce expression of VEGF to initiate angiogenesis (Dayan et al., 2008). Tumor angiogenesis involves several mechanisms of vascular growth including sprouting angiogenesis, intussusceptive growth, vascular co-option and vasculogenic mimicry. Tumor neovascularization also involves postnatal vasculogenesis, in which endothelial progenitor cells are recruited to the tumor (Donnem et al., 2013; Lyden et al., 2001; Maniotis et al., 1999; Moschetta et al., 2014; Rafii et al., 2002; Ribatti and Djonov, 2012; Seftor et al., 2012). Altogether, tumor angiogenesis results in poorly organized, tortuous and leaky vasculature that has loosely attached pericytes and abnormal BM (Abramsson et al., 2003; Baluk et al., 2003; Benjamin et al., 1999; Hashizume et al., 2000; Morikawa et al., 2002; Pries et al., 2010).

Vascular leakage is associated with interstitial hypertension, which can limit the delivery of antitumor agents into the tumor. Normalization of the tumor vasculature with anti-angiogenic drugs can thus increase blood perfusion and drug delivery to tumors (Jain, 2005). VEGF is overexpressed in most solid tumors. Thus, the development of anti-angiogenic drugs has been focused on the development of drugs targeting the VEGF pathway. Bevacizumab, which blocks VEGF function, is the first anti-angiogenic inhibitor that was approved for cancer therapy in 2004 (Ferrara and Kerbel, 2005). Bevacizumab, in combination with chemotherapy, improves the overall survival or progression-free survival in different cancer types such as ovarian cancer, cervical cancer, and metastatic colorectal cancer (Giantonio et al., 2007; Hurwitz et al., 2004; Perren et al., 2011; Tewari et al., 2014). However, in most other cancers, Bevacizumab has failed to increase survival (Jayson et al., 2016). Overall, anti-angiogenic therapies targeting the VEGF pathway thus far have had only modest efficacy. Treatment can be followed by tumor relapse, because of activation of alternative signaling pathways (Batchelor et al., 2007; Ebos and Kerbel, 2011; Kopetz et al., 2010). Thus, new combinatorial treatment strategies are needed for anti-angiogenic treatment of cancer.

Cancer immunotherapies, which restore the ability of the host immune system to recognize and destroy tumor cells, have shown great potential in cancer therapy (Miller and Sadelain, 2015; Palucka and Coussens, 2016; Sharma and Allison, 2015). Angiogenic factors have been shown to limit the function of anti-tumor T cells and thus drive an immunosuppressive tumor microenvironment. Targeting angiogenic signaling can enhance the potency of cancer immunotherapies (Huang et al., 2013; Lanitis et al., 2015; Motz and Coukos, 2011). This further supports the notion that new anti-angiogenesis therapies will be beneficial in the treatment of malignancies.

4.1.2 Ocular diseases

Ocular neovascularization in the retina, choroid, and cornea in response to pathological stimuli can lead to severe visual impairment. Ocular neovascularization is part of several disorders, including age-related macular degeneration (AMD), diabetic retinopathy (DP), retinal vein occlusion, retinopathy of prematurity and neovascular glaucoma. Ocular neovascularization is triggered by ischemia, which upregulates the production of angiogenic factors. Neovascularization can severely impair visual function due to hemorrhage, retinal detachment, fibrosis, and vascular leakage, which results in edema (Bressler, 2009; Dreyfuss et al., 2015).

VEGF is the most critical regulator of ocular neovascularization and blocking VEGF has been very successful in preventing vision loss in AMD and macular edema. However, approximately 40% of patients with AMD exhibit a suboptimal treatment response, and patients still experience gradual vision loss. Thus, further research is needed to find new combinatorial treatment strategies (Ferrara and Adamis, 2016; Kim and D'Amore, 2012).

4.1.3 Inflammation

Acute and chronic inflammation are associated with changes in microvascular function as the endothelium participates in the generation of the inflammatory response. Proinflammatory cytokines induce vascular leakage, and the endothelium regulates leukocyte transmigration into tissues. Leukocyte recruitment is a significant component of inflammatory responses. Inflammatory cytokines induce activation of ECs and expression of leukocyte adhesion molecules. Type I activation of the endothelium in inflammation is fast and is mediated for example by histamine. Sustained inflammation relies on type II activation of ECs and is mediated by cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α (Pober and Sessa, 2014).

Leukocyte recruitment into the tissues occurs via paracellular or transcellular migration of leukocytes. The leukocyte adhesion cascade includes slow rolling, adhesion strengthening, intraluminal crawling, paracellular and transcellular migration, and migration through the basement membrane (Ley et al., 2007). A variety of leukocyte adhesion molecules mediate different steps of the leukocyte adhesion cascade. ECs express E-selectin and P-selectin, which are critical for leukocyte rolling (McEver, 2015). Integrins also participate in rolling and they mediate firm leukocyte adhesion. The arrest of leukocytes to the vessel wall is induced by chemokines and mediated by the interaction between leukocyte-expressed integrins and adhesion molecules expressed in the ECs. EC adhesion molecules include intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) (Ley et al., 2007; Sans et al., 1999). For example, in a mouse model of MS, very late antigen 4 (VL4, also known as α4β1) was shown to mediate leukocyte recruitment to the CNS by binding to VCAM1 expressed on ECs (Elices et al., 1990; Yednock et al., 1992). The adhered leukocytes transmigrate through ECs via paracellular and transcellular routes that involve several endothelial adhesion molecules, including PECAM1, the JAM family members, ICAM2, and VCAM1 (Garrido-Urbani et al., 2008; Ley et al., 2007; Thompson et al., 2001).

Vascular hyperpermeability is a typical feature of acute inflammation, when venular ECs transiently contract, generating paracellular gaps (Majno and Palade, 1961; Majno et al., 1961). The increased permeability that occurs in response to acute inflammation is reversed when inflammation resolves. However, in chronic inflammation, excess vascular permeability persists, which is accompanied by angiogenesis and microvascular remodeling (McDonald, 2001). Inflammation-induced microvascular leakage is commonly localized to the site of infection, and it is mediated by microbial factors and host cytokines. In some cases, such as during systemic bacterial infection, termed sepsis, the localized infection spreads to the blood circulation. Then vascular leakage persists and affects multiple organs simultaneously. Leaky microvasculature induces edema, impairs gas exchange in the lungs, and leads to poor perfusion in other organs. Loss of intravascular fluid can cause shock, multiorgan failure, and even death (Ware and Matthay, 2000).

Mechanisms of vascular leakage in acute inflammation entails remodeling of the actin cytoskeleton in order to promote EC contraction and disassembly of intracellular junctions for the formation of gaps between cells (Dejana et al., 2009; Vandenbroucke et al., 2008). Permeability is also regulated by internalization of VE-cadherin that leads to disruption of the endothelial cell-cell junctions (Dejana et al., 2008). A variety of signaling molecules are involved in inflammation-induced vascular leakage, including members of the VEGF and Ang-Tie pathways (Parikh, 2017; Yano et al., 2006).

4.2 Lymphatic dysfunction

Reactivation of lymphangiogenesis is induced in many pathological conditions, including inflammation and cancer. Lymphangiogenesis occurs at sites of infection and their associated draining lymph nodes. Lymphangiogenesis is also observed in several human inflammatory diseases, such as psoriasis and rheumatoid arthritis (Aebischer et al., 2014; Kim et al., 2012, 2014). Inflammation-induced expansion of the lymphatic vessels promotes the resolution of the inflammation by increasing fluid drainage and immune cell migration (Baluk et al., 2005; Huggenberger et al., 2010). However, in organ transplantation, increased lymphangiogenesis promotes antigen presentation in lymphoid organs, which can result in organ rejection (Kim et al., 2012; Nykänen et al., 2010).

In inflammation, lymphangiogenesis is induced by lymphangiogenic factors such as VEGFC produced by macrophages (Harvey and Gordon, 2012). The same lymphangiogenic factors also promote tumor and lymph node lymphangiogenesis (Alitalo and Detmar, 2012; Hirakawa et al., 2007; Karpanen et al., 2001), which facilitates the dissemination of metastatic tumor cells into the lymphatic vessels and lymph nodes (Karpanen et al., 2001; Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001). Tumor lymphangiogenesis is considered to have a dual role in tumor development since it can enhance tumor metastasis but also the anti-tumor response of the immune system (Fankhauser et al., 2017; Mandriota et al., 2001; Song et al., 2020; Vaahtomeri et al., 2017).

Impaired lymphatic function leads to the accumulation of fluid in tissues, resulting in lymphedema. Lymphedema is classified into primary lymphedema and secondary lymphedema. Primary lymphedema is a sporadic condition that results from mutations in genes involved in lymphatic development. Many of the mutations involve the VEGFC/VEGFR3 signaling system (Brouillard et al., 2014; Warren et al., 2007). These mutations have been described to affect, for example, genes encoding VEGFR3, VEGFC, transcription factor FOXC2 and CCBE1, which enhances VEGFR3 signaling (Alders et al., 2009; Connell et al., 2009; Jeltsch et al., 2014; Mellor et al., 2007). Secondary lymphedemas account for most of the lymphedema cases. They arise from damage to the lymphatic vessels caused by for example surgery, infection, or trauma. A common cause is breast cancer surgery, where concomitant removal of lymph nodes results in impaired lymphatic drainage of the upper limbs and lymphedema (Warren et al., 2007).

Interestingly, lymphatic malfunction has also been linked with fat accumulation in peripheral tissues. In mice deleted of one Prox1 allele, the dysfunction of lymphatic vessels led to obesity and inflammation (Harvey et al., 2005). Furthermore, lymphatic vessels are also required for mobilization of cholesterol from tissues, including the arterial wall (Lim et al., 2013; Martel et al., 2013). Thus, lymphatic dysfunction is also associated with obesity and cardiovascular disease, such as atherosclerosis.

4.3 Ang-Tie system in disease

4.3.1 Ang-Tie system in tumor angiogenesis and metastasis

In addition to its function in physiological angiogenesis, the Ang-Tie pathway acts in tumor angiogenesis and metastasis. Increased expression of Ang2 occurs in several human cancers (Goede et al., 2010; Helfrich et al., 2009; Saharinen et al., 2017; Scholz et al., 2016; Sfiligoi et al., 2003; Wang et al., 2014). Numerous preclinical studies using Ang2-deficient mice, Ang2 targeting antibodies or peptibodies have shown that Ang2 inhibition reduces tumor growth by decreasing EC proliferation and vessel sprouting, and by promoting vessel regression (Holopainen et al., 2012; Mazzieri et al., 2011; Nasarre et al., 2009; Oliner et al., 2004). Ang2 inhibition also leads to normalization of the remaining vasculature, which is associated with improved vascular integrity and attachment of pericytes (Falcón et al., 2009).

In contrast to Ang2, Ang1 blockade appears to be less effective in inhibiting tumor growth. However, vessel stabilization, induced by Ang2 blockade, was shown to be Ang1 dependent (Coxon et al., 2010; Falcón et al., 2009). While Ang2 overexpression leads to vascular leakage and lack of pericytes, increased Ang1 expression enhances vascular normalization, which improves blood perfusion and drug delivery (Coxon et al., 2010; Falcón et al., 2009; Reiss et al., 2009). Furthermore, increased Tie2 activation upon treatment with the VE-PTP inhibitor or ABTAA induced vascular normalization and improved response to cytotoxic drugs (Goel et al., 2013; Park et al., 2016).

Ang2 was shown to cooperate with VEGF in tumors that initiate growth by coopting existing vessels. In the presence of VEGF, Ang2 promotes angiogenesis, whereas in the absence of VEGF Ang2 induces vessel regression (Holash et al., 1999). Ang2 has been suggested to be responsible for the development of resistance to VEGF therapy. Indeed, anti-VEGF therapy was shown to be associated with increased expression of Ang2 and recruitment of TEMs (Rigamonti et al., 2014). Furthermore, in tumors, Ang2 promotes EC survival via Tie2 activation, thus restricting the beneficial effects of VEGF inhibition (Daly et al., 2013). Thereby several studies have combined Ang2 blockade with VEGF inhibition, which was shown to inhibit tumor angiogenesis and growth more effectively than single treatments (Brown et al., 2010; Hashizume et al., 2010). The dual targeting also promoted the conversion of pro-tumorigenic M2 macrophages towards the anti-tumorigenic M1 phenotype in several preclinical tumor models (Kloepper et al., 2016; Peterson et al., 2016; Rigamonti et al., 2014; Scholz et al., 2016). Additionally, the antitumor immunity elicited by the dual inhibition was further improved by PD-1 checkpoint blockade (Schmittnaegel et al., 2017; Di Tacchio et al., 2019).

Ang2 blockade also reduces tumor metastasis in mouse models due to improved vascular integrity (Holopainen et al., 2012; Mazzieri et al., 2011). Ang2 inhibition reduced tumor lymphangiogenesis and lymph node metastasis (Holopainen et al., 2012). The function of Ang2 in metastasis seems to be organ-specific as its deletion reduced metastasis in the lungs but increased metastasis in the liver (Im et al., 2013). In line with the role of vascular integrity in metastasis, Ang1 deletion leads to increased metastasis, whereas Tie2 activation by ABTAA decreased metastasis (Michael et al., 2017; Park et al., 2016).

4.3.2 Ang-Tie pathway in ocular neovascularization

Physiological vascular development of the eye is dependent on the Ang-Tie pathway, which is also involved in ocular and diabetes-associated vascular diseases (Campochiaro, 2015; Saharinen et al., 2017). Retinal neovascularization is a pathogenic feature of DP, retinopathy of prematurity, and retinal vein occlusion. In a model of oxygen-induced retinopathy, which mimics the features of human retinopathy of prematurity, Ang2 expression was induced in the ischemic retina. Furthermore, Ang2 was required for ischemia-induced retinal neovascularization, whereas Ang1 promoted vascular normalization and reduced leakage (Hackett et al., 2000, 2002; Lee et al., 2013). In this model, VEGF was shown to regulate Ang2 function since Ang2 induced retinal neovascularization together with VEGF, but under conditions of low VEGF levels, Ang2 promoted vessel regression (Oshima et al., 2005).

Increased Ang2 concentrations have been found in the vitreous body of patients with DP, which is the most common diabetic complication (Tuuminen et al., 2015). In mouse models of DP, Ang2 was shown to increase leakage and pericyte loss, which is one of the hallmarks of DP (Hammes et al., 2004; Ogura et al., 2017). In contrast, Cartilage oligomeric matrix protein (COMP)-Ang1 fusion protein, which is a stable and potent Tie2 agonist, normalized retinal vasculature (Cahoon et al., 2015). In addition, Ang2 blockade or Tie2 activation by ABTAA were shown to reduce the breakdown of the blood-retina barrier, which contributes to maintenance of the eye as an immune-privileged site (Park et al., 2017). Furthermore, VE-PTP inhibition, which activates Tie2, improved vascular integrity in different models of ocular neovascularization (Shen et al., 2014b).

Vascular leakage from the choroid vessels and choroidal neovascularization are key pathogenic features of neovascular AMD. COMP-Ang1 was shown to inhibit choroidal neovascularization and leakage after laser-induced choroidal neovascularization model, whereas Ang1 deletion had the opposite effect (Lee et al., 2014b). In line with this, Tie2 activation by ABTAA reduced vascular leakage and choroidal neovascularization and induced choriocapillary regeneration in the same model. Furthermore, Ang-Tie2 signaling was also shown to be necessary for the maintenance of choriocapillaries (Kim et al., 2019). Altogether, these results indicate that activation of Tie2 signaling is beneficial in the treatment of ocular diseases.

4.3.3 Ang-Tie pathway in inflammation

The endothelium is an essential regulator of vascular homeostasis. However, in pathological conditions, endothelial activation or dysfunction is associated with inflammation, vascular leakage, and increased leukocyte recruitment. The Ang-Tie pathway is crucial for the regulation of vascular stability. In inflammation, Ang1 promotes vascular stabilization, whereas Ang2 promotes vascular leakage in concert with proinflammatory cytokines (Saharinen et al., 2017) (**Figure 4**). Tie2 is essential for the maintenance of vascular integrity, even in basal conditions, at least in the lung vasculature (Frye et al., 2015).

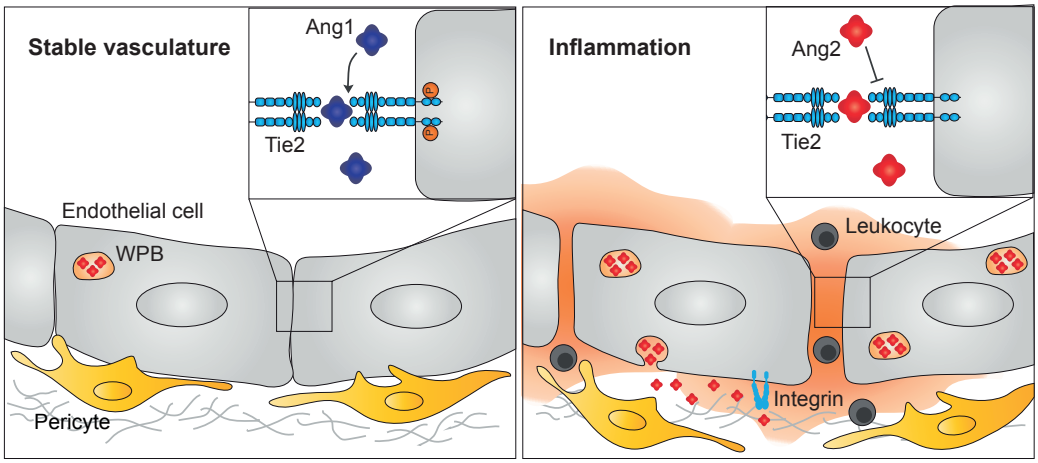

Figure 4. Angiopoietin signaling in vascular quiescence and inflammation. Pericyte expressed Ang1 promotes vascular stability via activation of the Tie2 receptor. In contrast, Ang2 promotes vascular leakage via antagonizing Tie2 activation and by signaling via integrins. WPB, Weibel-Palade body. Adapted from (Kiss and Saharinen, 2019).

In chronic inflammation, capillary-to-venous remodeling expands the vasculature to promote leukocyte recruitment and vascular leakage. One model to study chronic inflammation is *Mycoplasma pulmonis*-induced chronic infection of mouse airways. It is accompanied by vascular enlargement, increased expression of EC adhesion molecules and vascular leakage. In this model, Ang2 expression is increased and Tie2 phosphorylation is decreased (Tabruyn et al., 2010). Ang2 inhibition with antibodies inhibited the venous remodeling induced by *Mycoplasma pulmonis* infection. This was further enhanced by combinatorial treatment with TNF-α-targeting antibodies (Le et al., 2015). These data suggest that Ang2 is involved in inflammation-induced vascular remodeling.

In inflammation, the release of Ang2 from Weibel-Palade bodies is stimulated by several cytokines (Fiedler et al., 2004). Ang2 has a crucial role in the induction of inflammation by sensitizing ECs to the actions of proinflammatory cytokines (Fiedler et al., 2006). Increased Ang2 levels are detected in numerous inflammatory conditions, and Ang2 expression is correlated with poor prognosis in several diseases, including sepsis, acute respiratory distress syndrome (ARDS), and acute kidney injury (David et al., 2012; Kümpers et al., 2010; Parikh et al., 2006; Saharinen et al., 2017; Zinter et al., 2016). In contrast, expression of Ang1 and Tie2 are decreased in inflammation, resulting in an increased Ang2/Ang1 ratio and decreased Ang1-Tie2 signaling (Mofarrahi et al., 2008; Tabruyn et al., 2010). This promotes FOXO1 translocation into the nucleus, which further increases Ang2 expression (Ghosh et al., 2015).

Inflammation is often associated with increased vascular permeability. Although one study has suggested that Ang2 protects the endothelium in stressed conditions (Daly et al., 2006), several studies have shown that inhibition or genetic deletion of Ang2 inhibits vascular leakage and reduces leukocyte infiltration in mouse models of inflammation (Benest et al., 2013; Lee et al., 2018; Tabruyn et al., 2010; Ziegler et al., 2013). In line with this, Ang2 inhibition also increased survival in mouse models of sepsis and systemic inflammation (Stiehl et al., 2014; Ziegler et al., 2013). In contrast, Ang1 has been shown to protect the vasculature from inflammation-induced leakage and increase survival in a mouse model of sepsis (David et al., 2011; Thurston et al., 1999,

2000). Furthermore, activation of Tie2 via VE-PTP blockade has been shown to decrease leakage (Frye et al., 2015). Interestingly, convertion of Ang2 into a Tie2 agonist by using ABTAA was more beneficial than only blocking Ang2 function in a mouse sepsis model (Han et al., 2016). Recently, Tie2 activation via platelet-secreted Ang1 was shown to prevent leakage during leukocyte extravasation (Braun et al., 2020). In addition, Tie2 activation inhibited microvascular thrombus formation during systemic inflammation (Higgins et al., 2018).

Further underlying the importance of Tie2 in the endothelial barrier function, genetic variants influencing Tie2 expression have been shown to correlate with disease susceptibility. Patients with haplotypes associated with the lowest Tie2 expression had 31% higher ARDS risk, whereas patients with haplotypes associated with the highest Tie2 had a 28% reduction in ARDS risk. In line with this, mice with only one Tie2 allele had more severe vascular leakage, and higher mortality in sepsis mouse models (Ghosh et al., 2016). Furthermore, reduced expression of Tie receptors was found to be associated with susceptibility to Ebola hemorrhagic fever in mice (Rasmussen et al., 2014).

When Tie2 levels are low, Ang2 has been suggested to function via α5β1-integrin (Hakanpaa et al., 2015). In line with this, and similarly as in Ang2 inhibition, β1-integrin blockade was shown to inhibit LPS-induced vascular leakage (Hakanpaa et al., 2018; Stiehl et al., 2014). Tie1, Ang2, and α5β1-integrin have also been associated with atherosclerosis. Atherosclerotic lesions develop at sites of turbulent blood flow, and Tie1 and Ang2 are expressed at sites of non-laminar flow in the aorta in mice (Porat et al., 2004; Tressel et al., 2007). In mice, Ang2-deficiency was shown to reduce atherosclerosis progression during the early phase of the disease (Theelen et al., 2015). Tie1 has been shown to regulate the expression of EC adhesion molecules *in vitro* and in a mouse model of atherosclerosis, Tie1-deficiency reduced endothelial inflammation and decreased atherosclerotic lesions (Chan and Sukhatme, 2009; Chan et al., 2008; Woo et al., 2011). Furthermore, α5β1-integrin was shown to promote inflammation in a mouse model of atherosclerosis (Al-Yafeai et al., 2018; Yun et al., 2016).

4.3.4 Mutations affecting the Ang-Tie pathway

Venous malformations (VMs) are composed of dilated vein-like channels that have irregular, smooth muscle cell coating. Somatic mutations in the *Tek* gene, which encodes the Tie2 protein, cause more than half of the sporadically occurring unifocal VMs (Limaye et al., 2009; Vikkula et al., 1996). In addition, mutations affecting *PIK3CA*, which encodes the catalytic subunit of PI3K downstream of Tie2, have been described in VMs (Castel et al., 2016; Castillo et al., 2016). In addition, two somatic Tie2 mutations cause Blue rubber bleb nevus syndrome characterized by numerous VMs (Soblet et al., 2017). In contrast, loss-of-function mutations in Tie2 or Ang1 have been associated with primary congenital glaucoma (PCG), which causes childhood blindness. PCG results from defects in the development of Schlemm's canal and the trabecular meshwork (Aspelund et al., 2014; Souma et al., 2016; Thomson et al., 2017). These defects underlie the importance of balanced Tie2 signaling. Thus far, mutations affecting Tie1 have not been described.

AIMS OF THE STUDY

The aim of this study was to further elucidate the function of the Ang-Tie pathway in physiological and pathological angiogenesis and lymphangiogenesis. More specifically, we sought to investigate the role of Tie1 in lymphatic and blood vascular development and the function of Tie1 and Ang2 in inflammation. Furthermore, we wanted to understand how Tie1 modulates Ang-Tie2 signaling.

The specific aims of this study were:

I To study the function of Tie1 in embryonic lymphatic vessel development

II To elucidate the role of Tie1 in postnatal and pathological angiogenesis

III To clarify the function of Tie1 in Ang-Tie2 signaling during vascular remodeling and inflammation

IV To study the function of Ang2 in neuroinflammation

MATERIALS AND METHODS

The materials and methods used in this study are described in detail in the original publications. Tables and description of the most relevant materials and methods used in this thesis are provided below.

1. MATERIALS

Table 1. Mouse lines

Mouse line	Description	Reference	Used in
Cdh5iCreER^{T2}	Inducible Cre recombinase under the control of the *Cdh5* gene promoter	(Wang et al., 2010)	II, III
Cdh5-tTA	Tetracycline-controlled transactivator (tTA) under the *Cdh5* promoter	(Sun et al., 2005)	III, IV
FLP	*FLP* recombinase used to remove neomycin resistance cassette (*neo)* flanked by FRT sites	Jackson laboratory	I
PGK-Cre	Cre recombinase under the control of the *PGK* gene promoter	(Lallemand et al., 1998)	II
PdgfbiCreER^{T2}	Inducible Cre recombinase under the control of the *Pdgfb* gene promoter	(Claxton et al., 2008)	II, III
Rosa26iCreER^{T2}	Inducible Cre recombinase under the control of the *Rosa26* gene promoter	(Vooijs et al., 2001)	II
ROSA26-R	ß-galactosidase under the control of *Rosa26* promoter	(Soriano, 1999)	II
TetO-Ang2	Mouse Ang2 cDNA under the control of the Tet operator (TetO)	(Holopainen et al., 2012)	III, IV
Tie1$^{+/LacZ}$, *Tie1$^{LacZ/LacZ}$*	*Tie1* gene knockout; ß-galactosidase inserted into the *Tie1* gene	(Partanen et al., 1996)	I, II, III
Tie1$^{neo/neo}$	*Tie1* gene knockout; *neo* cassette inserted into the *Tie1* gene	(D'Amico et al., 2010)	I
Tie1$^{flox/flox}$	Conditional *Tie1* gene knockout; LoxP sites inserted into the *Tie1* gene	(D'Amico et al., 2010)	I, II, III
Tie1-tTA	Tetracycline-controlled transactivator (tTA) under the *Tie1* promoter	(Sarao and Dumont, 1998)	III

Table 2. Cell lines

Cell line	Description	Source	Used in
B16F1	Murine melanoma cell line	Caliper Life Sciences	II
B16F10	Murine melanoma cell line	Caliper Life Sciences	II
EL4	Murine lymphoma/leukemia cell line	A gift from S. Jalkanen	II
HUVEC	Human umbilical vein endothelial cell	PromoCell	III
LLC	Murine lung carcinoma cell line	ATCC	II

Table 3. Methods summary

Method	Used in
AAV transduction of mice	II
Adenoviral transduction of mice	III
Analysis of vascular leakage	III, IV
Analysis of vascular perfusion	II
Analysis of hypoxia	II
Cell culture and stimulations	II, III
Cell transfections	III
EAE mouse model	IV
FLIM and FRET	III
Flow cytometry	IV
Image analysis	I, II, III, IV
Immunofluorescence staining of coverslips	III
Immunohistochemistry of sections	I, II, IV
Immunoprecipitation and western blotting	II, III, IV
LPS induced endotoxemia in mice	III
Mice and treatments	I, II, III, IV
Microscopy	I, II, III, IV
Real-time quantitative PCR	II, III, IV
Single-cell RNA-sequencing	IV
Statistical analysis	I, II, III, IV
Transmission electron microscopy	II
Tumor experiments	II
TUNEL method for detection of apoptotic cells	II
Whole-mount staining	I, II, III
X-gal staining	I, II, III

Adenoviral transduction of mice

Adenoviruses encoding COMP-Ang1, Ang2, empty control, or LacZ were intravenously injected to the mice at a dose of $1x10^9$ plaque-forming units per mouse. COMP-Ang1 is a designed soluble and stable Ang1 variant (Cho et al., 2004). The mice were euthanized two days or two weeks after the injection of adenoviruses. The efficiency of transduction was analyzed from the lungs by western blotting.

Analysis of vascular leakage

Vascular leakage in the tracheal blood vessels was analyzed by injecting intravenously 100 µl of fluorescent 100 nm microspheres (Life Technologies) into mice treated with LPS. Adenoviruses encoding COMP-Ang1 were injected two days before LPS injection, and the leakage analysis was performed 16 hours after the LPS challenge. Fluorescent microspheres were allowed to circulate for 4 minutes, after which the mice were perfused with 10 ml of PBS or 10 ml of 1% PFA/PBS via the left ventricle and the tracheas were collected for analysis.

Vascular leakage in the spinal cord blood vessels was analyzed 12 days after immunization by injecting intravenously 100 µl of 3% Evans blue into control and EAE mice. Three hours later, mice were perfused with PBS, and spinal cords were collected for Evans blue extraction in deionized formamide at 56°C overnight. Absorbance was measured at 620 and 740 nm.

Analysis of vascular perfusion

For analysis of tumor vascular perfusion, 100 µl of biotinylated Lycopersicon esculentum (Tomato) lectin (1 mg/ml, Vector Laboratories) was injected intravenously into the tumor-bearing mice 10 min before sacrifice. Vessel perfusion was analyzed in frozen tumor sections by quantifying the percentage of lectin-positive vessel area per total vascular area in the microscopic field.

Analysis of hypoxia

Tumor hypoxia was analyzed by injecting intraperitoneally 60 mg/kg of pimonidazole hydrochloride (Hypoxyprobe kit, Millipore) to the tumor-bearing mice 1.5 hours before sacrifice. Visualization of the hypoxic area was performed by staining tumor frozen sections with a FITC-conjugated antibody that recognizes pimonidazole adducts in hypoxic cells (Hypoxyprobe-1 Mab1, Millipore). The hypoxic tumor area was calculated as a percentage of the total area of the microscopic field.

Cell culture and stimulations

B16F1 and B16F10 melanoma cells (Caliper Life Sciences), as well as Lewis lung carcinoma (LLC) cells (ATCC), were maintained in DMEM, supplemented with 2 mM L-glutamine, penicillin (100 U ml−1), streptomycin (100 µg ml−1) and 10% fetal calf serum (Promo Cell). EL4 leukemia/lymphoma cells (a gift from S. Jalkanen) were grown in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 1 mmol/L sodium pyruvate (Gibco), MEM vitamin solution (Gibco), penicillin (100 U ml−1), streptomycin (100 µg ml−1), and 10% fetal calf serum. Human umbilical vein endothelial cells (HUVECs) (PromoCell) were grown in Endothelial Cell Basal Medium MV (ECBM) with supplements provided by the manufacturer on 0.1% gelatin-coated culture plates or coverslips. For recombinant protein stimulations, HUVECs were starved for 3 to 5 hours in 1% fetal calf serum/ECBM and treated for 10-30 minutes with COMP-Ang1 (200-500 ng/ml), or Ang2 (R&D systems, 200-500 ng/ml).

EAE mouse model

Active EAE was induced in mice using the EAE induction kit following the manufacturer's instructions (Hooke Laboratories, EK-2210). Briefly, mice were injected subcutaneously into two sites on the back with 200 µg of myelin oligodendrocyte glycoprotein (MOG)35-55 in complete Freund's adjuvant followed by an intraperitoneal injection of 200-400 ng of pertussis toxin on the same day and the following day. Mice were monitored daily, starting from 7 days post-immunization (dpi) until 12, 14, or 28 dpi for clinical signs of paralysis.

Image analysis

Image analysis was performed using Image J/Fiji Image J software. The number of sprouting vessels on the pial side and the number of branchpoints on the subventricular side of hindbrains were counted manually (I). Tumor vasculature positive for endomucin or PECAM1 was quantified from images taken from peripheral and central regions and reported as area fraction (II). P-selectin and ICAM1 areas in tumor vessels were measured with ImageJ, normalized to endomucin$^+$ vessel area and indicated as percentage (II). The index of proliferating endothelial or tumor cells was quantified as the percentage of BrdU$^+$ ECs per endomucin$^+$ vessel area or the percentage of BrdU$^+$ tumor cells per total area in each microscopic field (II). The index of apoptotic endothelial or tumor cell death was calculated as percentage of TUNEL$^+$ or Caspase3$^+$ ECs per endomucin$^+$ vessel area

or the percentage of TUNEL$^+$ or Caspase3$^+$ tumor cells per total area in each microscopic field (II). The vascular surface area of the retinas was quantified from isolectin-B4 stainings from all intact quarters of the samples (II). Vessel branchpoints, sprouts and filopodia were counted manually from retina whole mounts and tumor sections (II). Average capillary and postcapillary venule diameter were measured manually from vessels over cartilage rings in each trachea (III). P-selectin and EphB4 areas in tracheal vessels were calculated using Fiji Image J, normalized to total PECAM1$^+$ vascular area and indicated as percentages (III). The number of proliferating ECs in the tracheas was calculated as area fraction of Ki67$^+$ ECs normalized to PECAM1$^+$ vascular area (III). Tie1 and Tie2 stainings in the vessels were analyzed by calculating the staining intensity per vessel (III). The number of CD11b$^+$ cells were counted manually and reported as area fraction of microscopic field (III). Leakage of microspheres was calculated as the percentage of microspheres per microscopic field (III). Stainings for phospho-Tie2, FOXO1, Ang2 and vWF in the tracheal vessels were calculated as area densities and normalized to PECAM1 (III). Iba1$^+$, CD4$^+$ and Ly-6G$^+$ immune cells were reported as area fraction of region of interest (IV). Vessel density and diameter (total vessel area divided by total vessel length) in the spinal cords were quantified from PECAM1 stainigs using Angiotool (III) (Zudaire et al., 2011). P-selectin$^+$ and VCAM1$^+$ area within the PECAM1$^+$ area in the spinal cords was reported as percentage (IV). MCHII$^+$ area within the Iba1+ area in the spinal cords was reported as percentage (IV). Extravasated TER-119$^+$ red blood cells were reported as percentage of TER-119$^+$ area outside the blood vessels normalized to PECAM$^+$ area (IV).

Immunohistochemistry of sections

After sacrificing the mice, the tissues were prepared for sections by immersing them in OCT or embedding them in paraffin. For frozen sections, the samples were first fixed in 4% PFA, washed with PBS, treated with 25-30% sucrose/PBS and embedded in OCT or the samples were alternatively embedded straight in OCT and frozen down in a bath of 2% pentane in isopentane on dry ice. For paraffin sections, the samples were fixed in 4% PFA, washed with PBS, dehydrated in a tissue processor and embedded in paraffin. Paraffin sections were first deparaffinized in xylene, rehydrated through graded ethanol series, and subjected to antigen retrieval. The following antigen retrieval methods were used: microwaving in citrate buffer (1.8 mM citric acid, 8.2 mM sodium citrate, pH 6.0), High pH buffer (S-2367, Dako or 10mM Tris base 1mM EDTA, 0.05% Tween-20, pH 10) or trypsin solution (0.1% trypsin, 0.1% CaCl2, pH 7.8). Frozen sections were dried at RT for 30 min-1 h and fixed with 1% PFA, cold methanol or acetone. Sections were permeabilized in PBS with 0.3% Triton X-100 and blocked in TNB, 5-10% normal donkey or goat serum in 1% (v/w) BSA in PBS, or donkey immunomix (5% donkey serum, 0.2% BSA, 0.3% Triton X-100, 0.05% NaN_3 in PBS) at RT for 1 h. Sections were incubated with primary antibodies in blocking solution at 4°C for one to two nights. After washing, sections were stained with fluorophore-conjugated secondary antibodies. After washing the sections were mounted in Vectashield mounting medium (Vecta Laboratories) containing DAPI or stained with DAPI. Alternatively, the endogenous peroxidase was quenched with 3% hydrogen peroxide and the primary antibodies were subjected to chromogenic staining by using the Tyramide Signal Amplification kit (Perkin Elmer) following the manufacturer's instructions. Following chromogenic staining, the sections were mounted in Permount (Fisher Scientific) or Aquamont (BDH Laboratory Supplies). Finally, the slides were sealed with Cytoseal (Thermo Scientific).

Immunoprecipitation and western blotting

Embryos and postnatal and adult lungs were lysed in 0.5% TX-100 and 0.5% NP-40 in PBS or with RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors (1 mM PMSF, 1 mg/mL each aprotinin and leupeptin, 1 mM Na3VO4 and 1 mM NaF). The tissues were lysed using 1.4 mm Ceramic Bead Tubes (MO BIO Laboratories) and PowerLyzer® 24 homogenizer (MO BIO laboratories). Protein concentration was calculated using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of total protein per sample was used for western blotting or immunoprecipitation. For detection of proteins in serum, 2.5 µl of serum per sample was mixed with lysis buffer. For immunoprecipitation the samples were incubated with primary antibodies and protein G–Sepharose (GE Healthsciences Ab) or protein G/A–Sepharose (Santa Cruz Biotechnology) at 4^{oC}. The total lysates or immunocomplexes were separated in SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with primary and secondary antibodies. The immunoblots were developed with the SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Maximun Sensitivity Substrate (Thermo Scientific). Western blots were imaged with Odyssey FC (LI-COR) or captured on a film.

LPS-induced endotoxemia in mice

LPS-induced endotoxemia was induced by intraperitoneal injection of LPS (*E. coli* LPS 055:B5, Sigma-Aldrich) at a dose of 15 mg/kg in mice. Mice were euthanized at 0.5, 1, 3, 6, 12, or 16 hours after LPS challenge.

Mice and treatments

All mouse experiments were approved by Committee for Animal Experiments of the District of Southern Finland. Mice were anaesthetized with intraperitoneal injections of xylazine (10 mg/kg) and ketamine (80 mg/kg). The transgenic mice were genotyped using standard PCR protocols.

Cre-recombinase activation and Ang2 repression

Inducible inactivation of Tie1 was achieved by tamoxifen administration to transgenic mice, which results in Cre-recombinase activation. To study embryos, 2 mg of 4-hydroxytamoxifen (4-OHT) in sunflower oil was administered to pregnant females at E7.5 and E8.5 (II). For pup studies, pups were injected with 50 µg of 4-OHT dissolved in ethanol at P1-P4 (II). For adult studies, adult mice received administration of tamoxifen in corn oil by oral gavage (2 mg/mouse/d) for 5 consecutive days or 3 times per week for a total of 3-8 times (II, III). For tumor experiments, mice were subcutaneously implanted with slow release (21 days) tamoxifen (free base, 15 mg) pellets (II). Ang2 overexpression in transgenic mice was repressed until birth by administration of 2 mg/ml of tetracycline in 5% sucrose in the drinking water of pregnant females or by giving them doxycycline-supplemented food (ssniff Spezialdiäten GmbH, Germany). Efficient Tie1 deletion was validated by Southern blotting, PCR genotyping, Northern blotting, qRT-PCR and Western blotting. Ang2 overexpression was confirmed by qRT-PCR and ELISA. Genotypes were confirmed by PCR.

DAPT treatment

Pharmacological Notch inhibition was induced by injecting vehicle or DAPT (Sigma) subcutaneously into the postnatal pups every 12 h and starting 48 h before analysis.

Antibody treatments

To study postnatal retinal angiogenesis, control and Ang2 antibodies (Holopainen et al., 2012; Leow et al., 2012) were injected subcutaneously at a dose of 30 mg/kg to postnatal pups at P1-P4. In EAE experiments, mIgG1 isotype control antibody (Eli Lilly and Company), mouse anti-mouse Ang2 antibody (Ang2 antibody, 18E5, Eli Lilly and Company), hIgG1 isotype control antibody (Synagis, AbbVie), and ABTAA (Han et al., 2016) were injected intraperitoneally at a dose of 20 mg/kg body weight two to three times a week for the duration of the experiment.

Growth factor stimulations

Growth factors hVEGF (gift from Dr. Michael Jeltsch), COMP-Ang1 and hAng2 (Anisimov et al., 2013) were injected intravenously in 100 µl of PBS at a dose of 10-20 µg per mouse and allowed to circulate for 10 min.

Microscopy

Fluorescently labeled whole mount samples were imaged with a confocal microscope Zeiss LSM 510, LSM 780 or LSM 880 (Carl Zeiss) in multichannel mode. Fluorescently labeled sections were imaged with Axio Imager (Carl Zeiss) or confocal microscopy. Chromogen stainings were imaged using transmitted light microscope (Leica). Images were edited using Photoshop or Fiji ImageJ.

Real-time quantitative PCR

Total RNA from tissues was isolated using TRIsure (Bioline) and the NucleoSpin RNA II Kit (Macherey-Nagel). Total RNA was reverse-transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) or the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR (RT-qPCR) was carried out using the iQ Supermix (Bio-Rad), the SYBR Green Supermix (Bio-Rad) or FastStart Universal SYBR Green Master Mix (Roche). RT-qPCR was performed on the BIO-RAD C1000 Thermal cycler or the CFX384 Real-Time PCR Detection System (Bio-Rad) using standardized protocols. Fold changes were calculated using the comparative CT (threshold cycle) method.

Single-cell RNA-sequencing

$CD45^+$ immune cells and $CD31^+$ ECs were enriched from spinal cords by using the Neural Tissue Dissociation Kit (P) and Myelin Removal Beads II (Miltenyi Biotec). Viable $CD45^+$ immune cells and $CD31^+$ ECs were sorted with BD Influx Cell Sorter (BD Biosciences) into PBS with 0.04% BSA. Approximately 10,000 sorted cells were further processed for single-cell RNA-sequencing scRNA-seq) using Chromium Single Cell 3' Reagent Kit v2 and Gel Bead Kit v2 (10×Genomics) according to the manufacturer's instructions. Sequencing was performed with a Novaseq 6000 Sequencing System (Illumina) and raw data was processed with the Cell Ranger analysis pipelines. scRNA-seq data were analyzed with the Seurat package 2.3 in R.

Statistical analysis

Statistical analysis was carried out with SPSS or Prism. A *P* value of less than 0.05 was considered to be statistically significant.

Tumor experiments

Syngeneic tumor grafts were done by subcutaneously injecting 1–3 × 10^6 tumor cells into abdominal flanks of control and Tie1-deleted mice. The tumor growth rates were followed by measuring the width (w), height (h) and depth (d) of the subcutaneous tumors every second day

with a digital caliber. Tumor volumes were calculated using the formula w x h x d/2. For combinatorial experiments, placebo, control, anti-VEGF (mrc84, 200 µg), and anti-VEGFR2 (DC101, 800 µg), were injected intraperitoneally four days after the tumor cell implantations. Injections were done every 3.5 days (mrc84) or every 2 days (DC101) for the duration of the experiment. In order to induce systemic overexpression of mouse Tie2 extracellular domain (mTie2-ECD) or mFc, adeno-associated virus (AAV) serotype 9 encoding mTie2-ECD or mFc was injected intraperitoneally at the dose of $1x10^{12}$ viral particles per mouse. LLC tumor cells were implanted one day after the AAV injections. mTie2-ECD expression was analyzed from the serum by western blotting five days after AAV injections.

Whole mount staining

For whole mount stainings, the tissues were fixed in 1-4% PFA for 1 h at RT. The tissues were blocked in donkey immunomix (5% donkey serum, 0.2% BSA, 0.3% Triton X-100, 0.05% NaN_3 in PBS) for 1-2 h and incubated in primary antibodies, diluted in the blocking buffer, at +4°C for 1-3 days or overnight at RT. The samples were washed with 0.3% Triton X-100 in PBS and stained with fluorophore-conjugated secondary antibodies for overnight at 4°C or RT. The samples were washed again with 0.3% Triton X-100 in PBS, post-fixed with 1-4% PFA, washed with PBS, and mounted with Vectashield mounting medium (Vector labs).

RESULTS AND DISCUSSION

Loss of endothelial Tie1 receptor impairs lymphatic vessel development (I)

Tie1 deletion leads to embryonic lethality starting from E13.5 due to severe hemorrhage and edema. Major blood vessels of the Tie1 knockout embryos appeared normal, but the microvasculature was disrupted, suggesting that Tie1 was required for the maintenance and survival of capillaries (Puri et al., 1995). Edema is a typical manifestation of lymphatic dysfunction. Thus, the edema observed in the Tie1 deleted embryos prompted us to investigate the function of Tie1 in the development of lymphatic vessels.

Tie1$^{-/-}$ embryos show edema, but no overt blood vessel defects at E12.5

For our studies, we used Tie1 knockout (*Tie1$^{-/-}$)* embryos in pure ICR genetic background. We found that the embryos survived until E14.5, which was longer than in the reported mixed 129Sv/CD-1 background (E13.5) (Puri et al., 1995). We first analyzed *Tie1$^{-/-}$* embryos from E11.5 until E14.5. At E11.5, the embryos appeared normal, whilst at E12.5, the embryos displayed swelling but no hemorrhage (**I, Fig. 1A**). We confirmed that the *Tie1$^{-/-}$* embryos had a normal blood vasculature at E12.5 by detailed comparison of the control and *Tie1$^{-/-}$* embryos. Whole mount staining of the entire embryo revealed no blood vessel abnormalities in the Tie1-deleted embryos (**I, Fig. S1**). Blood vessel density and pericyte coverage were normal in the hindbrains, and SMA coverage in the carotid artery of *Tie1$^{-/-}$* embryos was similar to that in the controls (**I, Fig. S2**). Since there was no loss of blood vessel integrity, the observed swelling was likely to result from defective lymphatic vessel development. When analyzed at E13.5, the embryos had developed both hemorrhages and edema (**I, Fig. 1A)**.

Tie1-deficiency results in fragmented lymphatic sacs

The appearance of edema prior to defects in blood vasculature suggested a role for Tie1 in lymphatic development. In line with this, ß-galactosidase staining in heterozygous *Tie1$^{+/-}$* embryos (*LacZ* inserted into the *Tie1* locus), revealed robust Tie1 expression in developing lymph sacs (**I, Fig. S3)**. Furthermore, *Tie1$^{-/-}$* embryos displayed abnormally patterned and fragmented lymph sacs starting from E12.5 (**I, Fig. 1B)**. We next generated Tie1 conditional knockout mice in which a neomycin resistance cassette was inserted into the first intron of the Tie1 gene, and exon1 was flanked by loxP sites to allow for Cre-recombinase mediated excision (**I, Fig. 2A**). These hypomorphic *Tie1$^{neo/neo}$* embryos showed edema without signs of hemorrhage at E15.5 (**I, Fig. 2B**), and they had normal blood vessels as analyzed by staining of sections at different levels of the thoracic region. Although the *Tie1$^{neo/neo}$* embryos had no hemorrhages, a subset of blood vessels appeared congested with blood, perhaps because of secondary to edema or intravascular blood clots (**I, Fig. S5**). Similar to the *Tie1$^{-/-}$* embryos, the hypomorphic *Tie1$^{neo/neo}$* mutants displayed abnormally patterned lymph sacs (**I, Fig. 2C and S6**).

Tie1 in lymphatic vessel development

In line with our work, Qu et al also showed that Tie1 deletion results in abnormal lymphatic vessel development (Qu et al., 2010) and subsequent studies have revealed that Tie1 is essential for lymphatic valve and collecting vessel development in both embryos and in postnatal mice (Qu et

al., 2015; Shen et al., 2014a). Our study focused on analysis of the function of Tie1 in the development of the lymph sacs, which arise from the venous endothelium (Srinivasan 2007). These structures give rise to the lymphatic network through sprouting lymphangiogenesis. Our work reveals that Tie1 is critical in these early developmental stages, as it is expressed in lymph sacs and its deletion results in fragmented lymph sacs. Interestingly, recent studies indicate that some lymphatic vascular beds have a nonvenous origin (Ulvmar and Mäkinen, 2016). It is currently unknown whether Tie1 is important for the development of lymphatic vessels arising from such an alternative origin. Thus, future studies are needed to shed light to the functions of Tie1 in organ-specific lymphatic vasculatures.

Our study showed that lymphatic defects precede blood vascular defects in the Tie1 mutant mice, suggesting that lymphatic vessels are more sensitive to loss of Tie1 than blood vessels. This is in contrast to other members of the Ang-Tie pathway as the deletion of either Ang1 or Tie2 leads to embryonic lethality due to severe defects in the blood vasculature (Augustin et al., 2009). On the other hand, Ang2 overexpression leads to a similar phenotype as Ang1 or Tie2 deletion, indicating that Ang2 negatively regulates Ang1-Tie2 signaling in the blood vessels (Maisonpierre et al., 1997). However, Ang2-deleted mice show prominent lymphatic defects after birth as well as impaired regression of hyaloid vessels and reduced sprouting angiogenesis in the developing retina (Dellinger et al., 2008; Gale et al., 2002). Interestingly, the Tie2 agonist Ang1 could only rescue the lymphatic defects caused by Ang2 deletion, indicating that Ang2 acts as a Tie2 agonist in the lymphatic vasculature (Dellinger et al., 2008; Gale et al., 2002). These results suggest that during development, both Tie1 and Ang2 have a prominent role in the lymphatic vasculature, whereas Tie2 and Ang1 are more important for blood vessel development.

Recent studies using inducible Tie2 conditional knockout mice, enabling its deletion at later stages, indicate that also Tie2 is involved in embryonic lymphatic vessel development (Souma et al., 2018). Interestingly, Tie2 activation in BECs vs LECs was shown to be differentially regulated by the tyrosine phosphatase VE-PTP. This study indicated that the lack of VE-PTP in lymphatic vessels enables the weak agonist Ang2 to efficiently promote Tie2 activation in LECs (Souma et al., 2018). However, it is not known how lack of VE-PTP affects the Tie1-Tie2 signaling. Future studies using Tie1 and Tie2 double inducible knockout mice would be required to gain new insight into the interplay between Tie1 and Tie2 in the lymphatic vasculature.

Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy (II)

Compounds targeting the VEGF/VEGFR2 pathway are in clinical use for cancer treatment (Ivy et al., 2009), but the therapeutic benefit is often insufficient. Thus, novel approaches are needed for cancer treatment (Singh and Ferrara, 2012). Ang2 levels are increased in many cancers, and in mice, Ang2 blockade inhibits tumor growth and metastasis (Holopainen et al., 2012). The expression of Tie1 in the adult vasculature is increased during vascular growth in physiological processes and in tumor angiogenesis (Kaipainen et al., 1994; Korhonen et al., 1992). In this study, we sought to investigate the function of Tie1 in tumor angiogenesis and growth, using Tie1 conditional knockout mice (I).

Tie1 deletion results in disruption of the longitudinal lymphatic vessel and primordial thoracic duct development

First, we validated our conditional knockout mouse model by deletion of Tie1 using the ubiquitously expressed PGK-Cre; this resulted in embryonic edema and hemorrhages, a phenotype resembling that reported in mice with a global knockout of Tie1 (**II, Fig. S1**) (Puri et al., 1995). For a more detailed analysis of the lymphatic vasculature, we used planar illumination-based ultramicroscopy in embryos in which Tie1 was deleted only from the ECs (*Tie1*$^{iEC\Delta/\Delta}$). PLLV and pTD, the earliest lymphatic structures emerging during development, appeared dilated in the Tie1-deleted embryos. Also, the superficial lymphatics that extend from the PLLV were disrupted (**II, Fig. S1B**). These data were in agreement with our previous report indicating a key role for Tie1 in lymphatic development (I) and they validated that Tie1 could efficiently be deleted in our conditional knockout model.

Tie1 deletion inhibits tumor growth and angiogenesis due to increased EC apoptosis

We next generated an EC-specific Tie1 conditional knockout mouse model, in which we could delete Tie1 in the endothelium of adult mice using tamoxifen. To investigate Tie1 function in tumor angiogenesis, we injected control and conditionally Tie1 deleted mice with tumor cells (Lewis lung carcinoma LLC, B16F10 melanoma, and EL4 leukemia/lymphoma cells) and assessed tumor growth and tumor vasculature development. We found that the tumor growth was significantly reduced in Tie1-deleted mice (**II, Fig. 1C and D**). Analysis of the tumor vasculature revealed that Tie1 deletion reduced blood vessel density and the number of vessel sprouts and filopodia (**II, Fig. 2**). To study the reasons for the reduced vessel area, we investigated apoptosis markers by immunostaining, as Tie1 has been shown to be crucial for EC survival in embryos (Kontos et al., 2002; Partanen et al., 1996). Indeed, apoptosis was increased in the tumor vessels of the Tie1-deleted mice, whilst no difference was observed in EC proliferation (**II, Fig. 3A-F and S6**). Further analysis revealed less perfused tumor vessels, increased tumor cell apoptosis, and a trend towards increased tumor hypoxia after Tie1 deletion (**II, Fig. 4**). These results indicate that Tie1 has a crucial role in promoting tumor angiogenesis and growth.

Tie1 deletion decreases the expression of EC adhesion molecules

In other models, Tie1 has been shown to promote inflammation (Chan and Sukhatme, 2009; Chan et al., 2008; Woo et al., 2011). In line with this, expression of EC adhesion molecules P-selectin

and ICAM1 was reduced in the vessels of Tie1-deleted mice (**II, Fig. S8**). However, this was not associated with differences in leukocyte (CD45), monocyte/macrophage (f4/80), or granulocyte (Lys6/Gr-1) infiltration (**II, Fig. S9**), suggesting that immune cells may not have a significant impact in the decreased tumor growth in Tie1-deleted mice.

Tie1 deletion does not affect the healthy vasculature

As Tie1 deletion led to increased apoptosis in the tumor vasculature, we next analyzed the effects of Tie1 deletion in healthy vasculature. After three weeks of deletion in adult mice, no differences were observed in the vascular densities in various organs (**II, Fig. S10A and B)**. Furthermore, analysis revealed no changes in vascular integrity in the tracheal vessels or in the lungs (**II, Fig. S10C-M)**. Moreover, Tie1 deletion did not affect the ultrastructure of the capillaries in the kidney glomerulus or kidney function (**II, Fig. S11)**. These results indicate that Tie1 deletion affects only the tumor vasculature and not healthy vessels.

Tie1 deletion does not improve anti-angiogenic therapy targeting VEGF or VEGFR2

Ang2 blockade has been demonstrated to improve the efficacy of anti-angiogenic treatments targeting VEGF/VEGFR2 (Brown et al., 2010; Hashizume et al., 2010; Koh et al., 2010). Having observed that Tie1 deletion inhibits tumor growth and angiogenesis, we asked if Tie1 deletion has synergistic effects when combined with current anti-angiogenic therapies. Thus, we combined Tie1 deletion with VEGF or VEGFR2 blocking antibodies in our *in vivo* model and assayed for tumor growth. Although Tie1 deletion was equally effective as VEGF or VEGFR2 blockade in inhibiting tumor growth, simultaneous targeting of Tie1 and VEGF or VEGFR2 did not result in an additive effect (**II, Fig. S12)**. Interestingly, we detected reduced VEGFR2 protein in the Tie1-deleted mice after VEGF injection, which might explain the lack of synergistic effects (**II, Fig. S13)**. Reduced VEGFR2 levels may also contribute to the increased apoptosis of ECs in the tumor vasculature of the Tie1-deleted mice, as VEGFR2 has been shown to be important for EC survival (Koch and Claesson-Welsh, 2012).

Effect of Tie1 on Tie2 signaling

Next, we investigated the function of Tie1 in Ang-Tie2 signaling, since this pathway is implicated in tumor growth. Previous *in vitro* studies have shown that Tie1 silencing increases Ang2-induced Tie2 activation, suggesting that Tie1 may act as a negative regulator of Tie2 (Seegar et al., 2010). However, we did not observe significant difference in Tie2 phosphorylation in Tie1-deleted mice. In contrast, we found that Tie1 deletion resulted in decreased Tie2 phosphorylation at Y1106 (**II, Fig. S15)**, which is a site for Dok-R protein binding that promotes Ang1-mediated EC migration (Jones et al., 2003). These data indicate that Tie1 promotes Ang1-mediated Tie2 activation *in vivo*.

Combination of Tie1 deletion and angiopoietin blockade results in additive tumor growth inhibition

Ang2 is the primary Tie2 ligand in tumors, and its blockade has been shown to inhibit tumor angiogenesis and growth (Holash et al., 1999). Thus, we next tested whether Ang2 blockade would synergize with Tie1 deletion to inhibit tumor growth. Since we did not find a tumor isograft model sensitive to anti-Ang2 antibodies, we transduced mice with adeno-associated viruses encoding a

soluble extracellular domain of Tie2 (mTie2-ECD). mTie2-ECD, which binds to and blocks both Ang1 and Ang2, inhibited tumor growth. Importantly, we found that Tie1 deletion combined with mTie2-ECD inhibited tumor growth more efficiently than either of the therapies alone (**II, Fig. 5)**. These data indicate that the inhibitory effect of Tie1 deletion on tumor growth can be further enhanced by the combination with angiopoietin blockade.

Tie1-deficiency inhibits postnatal retinal angiogenesis

To further dissect the molecular mechanisms of Tie1 in angiogenesis, we used postnatal retina as a model. At P5, Tie1 was expressed in the growing retinal vasculature, including the tip cells (**II, Fig. 6A and B)**, which are Tie2 negative (Felcht et al., 2012). Tie1 deletion in this model reduced angiogenesis in a dose-dependent manner. Whilst no difference was observed in the retinal vasculature in mice where Tie1 deletion was 80-85% complete, almost complete Tie1 deletion resulted in reduced angiogenesis, including occasional EC tufts. The Tie1-deleted pups had fewer filopodia and sprouts in the retinal vasculature, similarly as observed in the tumor vessels of Tie1-deleted mice (**II, Fig. 6 and S17)**.

Tie1 deletion results in activation of the Notch pathway in postnatal retinal ECs

In order to understand the mechanisms related to the reduced retinal angiogenesis in the Tie1-deleted pups, we studied the involvement of the Notch signaling pathway, which is known to restrict angiogenesis (Phng and Gerhardt, 2009). In the developing retina, the Notch ligand Dll4 is expressed mainly in the angiogenic tip cells, and it activates Notch signaling in the adjacent stalk cells to suppress their sprouting. Upon Tie1 deletion, we detected increased expression of Dll4 and Notch target genes (**II, Fig. 7A-E)**. Furthermore, the levels of the intracellular domain of Notch (NCID) were increased, indicative of increased Notch activity (**II, Fig. 7H**). To validate the involvement of the Notch pathway, we treated control and Tie1-deleted pups with the γ-secretase inhibitor DAPT, which inhibits Notch signaling. Upon DAPT treatment, both control and Tie1-deleted pups developed similar hypervascularization in the retinas, and this treatment also prevented the increase of NCID in the Tie1 deleted pups (**II, Fig. 7F-H)**. These data suggest that Notch activation contributes to the decreased vasculature in the Tie1-deleted mice. Overall, these results indicate that Tie1 deletion results in the activation of the Notch pathway, which is consistent with the reduced angiogenic sprouting.

Combination of Tie1 deletion and Ang2 inhibition results in additive inhibition of retinal angiogenesis

Ang2 expression is enriched in the tip cells of endothelial sprouts and its blockade inhibits retinal angiogenesis (Felcht et al., 2012; Holopainen et al., 2012; del Toro et al., 2010). Thus, we tested the combinatorial targeting of Tie1 and Ang2 in the retinal vasculature. As expected, Tie1 deletion and Ang2 blockade led to decreased retinal angiogenesis. Targeting of Tie1 and Ang2 simultaneously resulted in additive inhibition of vascular migration (**II, Fig. 8**). These results are in line with the reduced tumor growth observed after Tie1 deletion and angiopoietin blockade.

Tie1 in retinal and tumor angiogenesis

In this study, we found that Tie1 regulates postnatal retinal and pathological tumor angiogenesis. Tie1 deletion resulted in reduced numbers of sprouts and filopodia in the retinal vasculature, and similar effects were observed in the tumor vessels. A role for Tie1 in retinal angiogenesis and tumor growth has by now been corroborated by subsequent studies (La Porta et al., 2018; Savant et al., 2015).

Tie1 has previously been shown to modulate Tie2 signaling. In this study, we found that Tie1 deletion in the quiescent vasculature of adult mice did not affect total Tie2 phosphorylation, but reduced Tie2 phosphorylation at Y1106. Phosphorylated Y1106 is known to bind the Dok-R protein and promote Ang1-mediated EC migration (Jones et al., 2003). Therefore, our results suggest that Tie1 is a positive regulator of Tie2 activity in this context. However, Savant et al. found that Tie1 differentially regulated Tie2 in endothelial tip vs. stalk cells. Tie1 inhibited Tie2 cell surface presentation in tip cells and sustained Tie2 signaling in stalk cells (Savant et al., 2015). In line with the suppressive function of Tie1 on Tie2 in tip cells, Tie1 deletion was also shown to increase the percentage of Tie2 positive ECs in the angiogenic tumor vessels (La Porta et al., 2018). These results suggest that one of the mechanisms of how Tie1 deletion affects tumor angiogenesis is by increasing Tie2 activation, which is known to promote vascular quiescence. Overall, these results indicate that Tie1 has different functions in quiescent vs. remodeling vs. angiogenic ECs. The context-dependent role of Tie1 is further supported by a recent study demonstrating that a Tie1 function-blocking antibody regulated Tie2 activation differently in cultured ECs and ECs in the lungs of tumor-bearing mice. Tie1 blockade *in vitro* inhibited Ang1-induced Tie2 phosphorylation, whereas in *vivo* the antibody increased Tie2 phosphorylation (Singhal et al., 2020).

We found that Tie1 deletion in tumor vessels increased EC apoptosis, and Savant et al. showed that Tie1 deletion increased EC apoptosis in the retina (Savant et al., 2015). These results are in line with the role of Tie1 in EC survival (Kontos et al., 2002). Thus, increased EC apoptosis may also contribute to the reduced angiogenesis in Tie1-deficient mice. Interestingly, a recent study showed that Ang2 blockade increased radiation induced apoptosis of tumor ECs, leading to slower tumor growth and increased survival of the tumor-bearing mice (Kallio et al., 2020). Furthermore, we found that Tie1 deletion increased activation of the Notch pathway, which is known to reduce angiogenesis. These results indicated that the Notch pathway was at least partly involved in the inhibition of angiogenesis in the Tie1-deleted mice. This is consistent with previous work, which shows that increased activation of the Notch pathway inhibits tumor angiogenesis and growth (Williams et al., 2006).

Importantly, EC-specific deletion of Tie1 reduced tumor angiogenesis and growth similarly as blocking antibodies against VEGF or VEGFR2, suggesting that Tie1 targeting has therapeutic potential. Blockade of the VEGF/VEGFR2 pathway is used in the clinics to treat ocular diseases and cancers. VEGF blockade has been shown to prolong disease-free survival only in a few tumor types (Jayson et al., 2016). Furthermore, anti-Ang2 treatments for cancer have not shown beneficial effects in clinical trials (Hyman et al., 2018; Papadopoulos et al., 2016) and a peptibody that inhibits both Ang1 and Ang2 showed only limited benefit in cancer patients (Monk et al., 2016; Vergote et al., 2019). However, the Ang1/Ang2 peptibody in combination with PD-1 immune checkpoint blockade is currently in clinical trial (www.clinicaltrials.gov; NCT03239145). Tie1 deletion reduced angiogenesis in the retina and in tumors, and combinatorial targeting of Tie1

and angiopoietins led to additive effects. Recent studies have also shown that both Tie1 deletion and Tie1 function-blocking antibodies inhibit tumor metastasis (La Porta et al., 2018; Singhal et al., 2020). Furthermore, in healthy vasculature, Tie1 deletion did not cause adverse effects. These results suggest that Tie1 could be a potential therapeutic target in angiogenesis-related disorders, where it appears to have synergistic effects with angiopoietin blockers.

Tie1 controls angiopoietin function in vascular remodeling and inflammation (III)

The Ang-Tie system controls vessel remodeling and vascular integrity in inflammation. Ang1 promotes Tie2 activation and vascular stability, whereas Ang2 antagonizes Tie2 and can induce vascular leakage in inflammation (Eklund et al., 2017). The unique function of the Ang-Tie pathway in regulating vascular stabilization makes this pathway a possible therapeutic target in inflammatory conditions (Saharinen et al., 2017). However, little is known about the function of Tie1 receptor and the mechanisms regulating the context-dependency of Ang2. In this study, we aimed to analyze the contribution of Tie1 in angiopoietin signaling and function.

Angiopoietins increase the interaction of Tie1 and Tie2

To investigate the dynamics of Tie receptors in angiopoietin signaling, we studied the interaction of Tie1 and Tie2 using methods which detect short-range molecular interactions: fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM). Using FRET/FLIM, we found that Tie1 and Tie2 interacted in unstimulated HUVECs, and this interaction was enhanced by Ang1 and Ang2 stimulation, especially at cell-cell junctions (**III, Fig. 1C-F and S1A**). Ang1-mediated Tie2 activation has been shown to be modulated by α5ß1-integrin (Cascone et al., 2005). Therefore, we analyzed whether ß1-integrin also affects the Tie1-Tie2 interaction. ß1-integrin silencing inhibited Ang1- and Ang2-induced Tie1-Tie2 interaction and the translocation of Tie1 and Tie2 to cell-cell junctions (**III, Fig. 2A-B, S2 and S3**). In addition, α5- and ß1-integrin silencing inhibited Ang1-induced Tie1 and Tie2 phosphorylation and downstream signaling (**III, Fig. 2D-G and S4A**). These results indicate that Tie1 is involved in angiopoietin signaling through its interaction with Tie2, and this interaction is ß1-integrin-dependent.

Tie1 is required for angiopoietin-induced vascular remodeling

To investigate the function of Tie1 in angiopoietin signaling *in vivo*, we analyzed the effects of Tie1 deletion on angiopoietin-induced vascular remodeling, using the EC-specific conditional Tie1 knockout mice (I, II). Systemic overexpression of angiopoietins is known to induce vascular enlargement and remodeling of capillaries into venules with high leukocyte adhesion receptor expression (Fuxe et al., 2010; Kim et al., 2007; Tabruyn et al., 2010). We induced systemic overexpression of angiopoietins by using adenoviral vectors encoding COMP-Ang1 (Ad-CAng1) or Ang2 (Ad-Ang2) in control and Tie1-deleted mice. Ang1 and Ang2 overexpression induced enlargement of tracheal vessels, upregulation of venous markers and EC proliferation. These changes were markedly reduced in the Tie1-deleted mice (**III, Fig. 3-5**), indicating that Tie1 is necessary for the angiopoietin-induced vascular remodeling.

Tie1 deletion decreases Ang1-induced Tie2 signaling

To decipher the mechanisms behind Tie1 function in angiopoietin-induced vascular remodeling, we analyzed Tie2 phosphorylation and downstream signaling. Ang1-induced Tie2 phosphorylation is known to stimulate Akt-dependent nuclear exclusion of the FOXO1 transcription factor, which leads to the downregulation of genes involved in endothelial destabilization and growth control (Daly et al., 2004; Zhang et al., 2002). Treatment with Ad-CAng1 resulted in increased Tie2 and Akt phosphorylation, which were reduced in the Tie1-

deleted mice (**III, Fig. 6A**). Tie1 deletion also inhibited CAng1-induced nuclear exclusion of FOXO1 and downregulation of FOXO1 target genes, *Esm1* and *Angpt2* (**III, Fig. 6B-E**). These data indicate that Tie1 promotes Ang1-induced Tie2 activation and downstream signaling.

Tie1 is required for agonistic function of Ang2 on Tie2 in homeostatic conditions

In order to mimic the autocrine function of Ang2, we used a tissue-specific transgenic mouse model to overexpress Ang2 in the endothelium ($Ang2^{EC}$). As in the Ad-Ang2 treated mice, $Ang2^{EC}$ mice had enlarged tracheal postcapillary venules and capillaries (**III, Fig. 7A-C**). Tie2 phosphorylation was increased in the lungs of Ang2 overexpressing mice and *Esm1* was downregulated, similarly as after CAng1 treatment (**III, Fig. 7D and F**). These data indicated that the transgene-encoded autocrine Ang2 acted as a Tie2 agonist in the Ang2 overexpressing mice. Interestingly, Tie1 levels were increased in the lung lysates of Ang2 overexpressing mice (**III, Fig. 7E**). To investigate whether Tie1 regulates the agonistic function of Ang2 on Tie2, we deleted Tie1 in $Ang2^{EC}$ mice. The Ang2-induced increase in Tie2 phosphorylation was blocked in the Tie1-deleted mice (**III, Fig. 7F**). In line with this, Ang2-induced Tie2 phosphorylation and translocation to cell-cell junctions were reduced after Tie1 silencing in HUVECs (**III, Fig. 7G and S11B**). Tie1 silencing also led to increased Tie2 internalization after Ang1 stimulation (**III, Fig. 11C**). Altogether, these results indicate that Tie1 promotes the agonistic activities of both Ang1 and Ang2 on Tie2 activation.

Tie1 is cleaved in acute inflammation

Our data suggested that Ang2 can act as a Tie2 agonist in both Ang2 overexpressing mice and in HUVECs during basal conditions. However, in inflammatory conditions, Ang2 is known to act as a Tie2 antagonist, promoting vascular leakage (Scharpfenecker et al., 2005; Tabruyn et al., 2010). During inflammation, Ang2 expression is increased and levels of Tie2 and Tie2 activation are decreased (Mofarrahi et al., 2008). We next focused on the function of Tie1 in inflammation using acute LPS-induced inflammation in mice as a model. As expected, Tie2 staining in tracheal vessels was gradually reduced after LPS stimulation, with circa 40% reduction after 6 hours (**III, Fig. 8B and C**). In contrast, Tie1 staining was rapidly lost after LPS treatment, with circa 80% reduction after 1 hour (**III, Fig. 8A and C**). This rapid reduction of Tie1 staining was likely due to Tie1 cleavage, as previous *in vitro* work has indicated that inflammatory cytokines induce the cleavage of the extracellular domain of Tie1 (Singh et al., 2012; Yabkowitz et al., 1999). In line with this, we detected increased levels of cleaved Tie1 ectodomain in lung lysates and serum after LPS treatment (**III, Fig. 8F and S13A**). In addition to LPS treatment, also treatment with TNF-α induced Tie1 cleavage (**III, Fig. S14D**), in line with previous *in vitro* studies (Singh et al., 2012). However, TNF-α blockade in LPS treated mice failed to prevent Tie1 cleavage, indicating that additional cytokines are involved in LPS-induced Tie1 cleavage (**III, Fig. S14D**). Altogether, these data indicate that inflammation leads to Tie1 cleavage *in vivo*. Importantly, increased Tie1 cleavage was also detected in the serum of patients diagnosed with acute Puumala hantavirus disease compared to healthy controls (**III, Fig. 8G**). This indicates that increased Tie1 cleavage occurs also in human inflammatory disease.

Ang2 does not function as a Tie2 agonist in LPS-induced inflammation

Next, we analyzed how LPS-treatment affects angiopoietin function. Besides inducing Tie1 cleavage, LPS treatment reduced the mRNA levels of Tie1, Tie2 and Ang1 (**III, Fig. S13B and C**), whereas Ang2 expression was increased (**III, Fig. S16D**). Previous work has shown that during inflammation, Ang2 induces a FOXO1 driven positive feedback loop, thereby increasing Ang2 transcription (Ghosh et al., 2015). In line with this, we found that LPS triggered the release of Ang2 from WBPs in tracheal vessels, which induced the translocation of FOXO1 into the nucleus (**III, Fig. S15C-D and S16A-C**). However, despite increased Ang2 levels, Tie2 and Akt phosphorylation were reduced (**III, Fig. 8D and S15A-B**). Furthermore, LPS treatment abolished Tie2 phosphorylation in Ang2 overexpressing mice (**III, Fig. 9A and B**). These results indicate that although Ang2 levels are increased after LPS treatment, Ang2 does not act as a Tie2 agonist during inflamation.

Ang1 reduces inflammation-induced Tie1 cleavage

In contrast to Ang2, Ang1 is known to induce vascular stability by promoting Tie2 activation in inflammation (Eklund et al., 2017; Gamble et al., 2000). LPS treatment also reduced Tie2 phosphorylation in Ad-CAng1 treated mice (**III, Fig. 9C and D**), indicating that LPS-induced Tie1 cleavage contributed to the reduced Tie2 activation by Ang1. Overall, these effects mimic that of Tie1 deletion, suggesting that LPS treatment, which induced Tie1 cleavage, contributes to the loss of Ang2 agonistic activity on Tie2 and reduces Ang1-induced Tie2 activation. In our model, Ad-CAng1 treatment reduced LPS-induced vascular leakage and Ang2 expression (**III, Fig. S17**). Interestingly, we found that treatment with Ad-CAng1 also reduced Tie1 cleavage in LPS treated mice (**III, Fig. 9G-I**). These results suggest that Ang1-induced reduction in Tie1 cleavage could contribute to its vascular stabilizing effects. However, Ang2 overexpression in LPS-treated mice did not induce Tie2 activation nor did it prevent the loss of Tie1 (**III, Fig. 9A, B, E and F**), consistent with previous work showing that Ang2 functions as a Tie2 antagonist in inflammatory conditions (Eklund et al., 2017; Saharinen et al., 2017).

Tie1 in angiopoietin signaling during vascular remodeling and inflammation

In this study, we investigated the interplay of Tie1 and the angiopoietin-Tie2 signaling. Our data sheds new light on this complex pathway (**Figure 5**). In non-inflammatory conditions, Tie1 facilitates agonistic actions of Ang1 and Ang2 on Tie2. This is likely because of a direct interaction of Tie1 with Tie2, which requires ß1-integrin. During inflammatory conditions, reduced Ang1 expression and the release of Ang2 from WBPs, plus subsequent Tie1 cleavage are associated with reduced Tie2 phosphorylation and increased activation of the FOXO1 pathway, leading to increased expression of Ang2. Reduced Tie2 levels in inflammation may promote Ang2 signaling via α5β1-integrin, which leads to vascular destabilization (Hakanpaa et al., 2015, 2018). During inflammation Ang2 acts as a Tie2 antagonist. This switch into Tie2 antagonistic Ang2 activity may be in part governed by Tie1, as reduced Tie1 levels and/or increased Tie1 cleavage prevent Ang2 induced Tie2 activation. Similar results were obtained in a different model of inflammation, wherein Tie1 cleavage was increased by chronic *Mycoplasma pulmonis* infection (Kim et al., 2016).

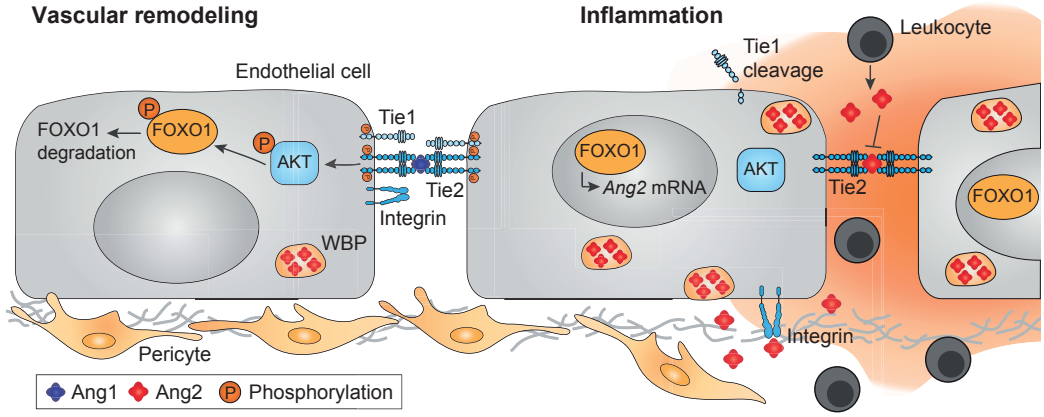

Figure 5. A model of Ang-Tie signaling during vessel remodeling and inflammation. Tie1 is required for angiopoietin-induced vascular remodeling via interaction with Tie2 in a β1-integrin dependent manner. In inflammation, cleavage of Tie1 is associated with loss of Ang2 agonistic activity on Tie2. Adapted from (Saharinen et al., 2017).

Overall, our results indicate that in both homeostatic and in inflammatory conditions, Tie1 is an essential component of angiopoietin-Tie2 signaling. These results are in line with work by Savant et al., in which they show that Tie1 sustains Tie2 signaling in remodeling endothelial stalk cells (Savant et al., 2015). In contrast, *in vitro* studies have suggested that Tie1 acts as a negative regulator of Tie2 signaling (Marron et al., 2007; Seegar et al., 2010; Yuan et al., 2007). These differences might be explained by the fact that Ang-Tie signaling is different at cell-cell junctions than at cell-substratum contacts (Fukuhara et al., 2008; Saharinen et al., 2008). Furthermore, Tie1 effects on Tie2 signaling in endothelial tip vs. stalk cells are different (Savant et al., 2015). These results indicate that the regulation of Tie2 signaling is context-dependent.

In contrast to Ang2, Ang1 appears to behave as an agonist of Tie2 in both inflammatory and non-inflammatory conditions. Kim et al. also showed that under non-inflammatory conditions, both Ang1 and Ang2 act as Tie2 agonists by promoting the formation of enlarged non-leaky vessels, but in *Mycoplasma pulmonis* induced chronic inflammation, Ang2 acts as a Tie2 antagonist (Kim et al., 2016). The Tie2-activating activity of Ang1 appears to be less affected by Tie1, likely because Ang1 is an intrinsically stronger agonist of Tie2 than Ang2. However, Tie1 deletion and Tie1 cleavage did reduce Ang1-induced Tie2 phosphorylation. Interestingly, Ang1 treatment is accompanied by reduced Tie1 cleavage in LPS-induced inflammation, suggesting that Ang1 inhibits Tie1 cleavage. Reduced Tie1 cleavage in the presence of increased Ang1 may thus contribute to Ang1 acting as a Tie2 agonist in inflammation. Overall, in inflammation Ang1 and Ang2 thus have opposing roles and this may be in part governed by their sensitivity to Tie1.

Tie1 cleavage has been shown to occur *in vitro* in response to various stimuli, including VEGF, TNF-α and IL-1β (Chen-Konak et al., 2003; Marron et al., 2007; Singh et al., 2012; Yabkowitz et al., 1999). Since TNF-α blockade did not rescue LPS-induced Tie1 cleavage in our model, other cytokines are likely involved as well. Reduced Tie1 levels and increased Tie1 cleavage has subsequently been reported in other inflammatory conditions, including a mouse model of myocardial ischemia and upon pericyte detachment from retinal vessels (Lee et al., 2018; Ogura et al., 2017; Park et al., 2017). Further studies, in which Tie1 cleavage is inhibited, are needed to clarify the function of Tie1 cleavage in inflammatory diseases. Furthermore, Tie1 cleavage also

results in the production of Tie1 intracellular domain with unknown functions in inflammation. Importantly, we detected increased Tie1 cleavage in patients suffering from an acute inflammatory disease, Puumala hantavirus infection. High baseline Tie1 levels were also recently shown to predict poor survival in patients with metastatic breast cancer (Tiainen et al., 2019). Overall, these results suggest that Tie1 could be used as a biomarker or therapeutic target in inflammatory diseases.

Angiopoietin-2 blockade ameliorates autoimmune neuroinflammation by inhibiting leukocyte recruitment into the CNS (IV)

The BBB is required for the maintenance of CNS homeostasis by restricting vascular permeability. Compromised BBB integrity, resulting in increased leukocyte infiltration, is a common pathological feature of several neurological disorders, such as inflammatory and neurodegenerative diseases (Larochelle et al., 2011; Obermeier et al., 2013). Ang2 is induced in several inflammatory diseases, including cancer and sepsis, and it promotes vascular leakage in these conditions (Saharinen et al., 2017). However, the role of Ang2 in CNS autoimmune diseases is not known. Here, we sought to investigate the involvement of Ang2 in CNS autoimmune disease by using transgenic Ang2 overexpressing mice in an EC-specific manner and Ang2 function-blocking antibodies.

Ang2 is induced in neuroinflammation and its overexpression exacerbates autoimmune neuroinflammation

EAE is a widely used mouse model to study MS, which is the most common human immune-mediated disorder affecting the CNS. To induce EAE, mice are injected with MOG_{35-55} peptide, leading to inflammation of the CNS, demyelination and progressive paralysis (Constantinescu et al., 2011). In order to study the function of Ang2 in neuroinflammation, we first measured Ang2 concentration in EAE mice. Their Ang2 concentration was increased in the serum at 7- and 21-days post-immunization (dpi) and in the spinal cords (SCs) at 14 and 28 dpi compared to untreated controls (**IV, Fig. 1A**), indicating induction of Ang2 expression in neuroinflammation. This is in line with studies showing elevated Ang2 levels in the brain and serum of MS patients (Chaitanya et al., 2013; Karampoor et al., 2016). To investigate if increased Ang2 expression could exacerbate EAE symptoms, we induced active EAE in control and transgenic Ang2 overexpressing mice ($Ang2^{EC}$). Indeed, $Ang2^{EC}$ mice exhibited higher EAE clinical scores and lost more weight than control mice (**IV, Fig. 1B**). These data suggest a role for Ang2 in neuroinflammation.

Ang2 blockade reduces autoimmune neuroinflammation

We reasoned that Ang2 blockade may modulate EAE. Thus, we injected mice with control or Ang2-blocking antibodies at the time of immunization (prophylactic treatment) or right before the onset of EAE symptoms at 7 dpi (pre-emptive EAE). In both cases, treatment with Ang2 antibodies reduced the severity of EAE symptoms (**IV, Fig. 1C and D**). In line with reduced EAE clinical scores, Ang2 antibody-treated mice had smaller demyelinated lesions in their (SCs) (**IV, Fig. 1E and F**). Based on the results from the pre-emptive experiment, Ang2 blockade seemed to affect the effector phase of the CNS autoimmune process and not only the T cell priming in the peripheral organs. To confirm this, we induced EAE by the adoptive transfer of differentiated effector T cells (adoptive transfer EAE). Also in this setting, Ang2 blockade ameliorated EAE severity (**IV, Fig. 1G**). In addition, we found that Ang2 blockade reduced infiltration of effector T cells into the SCs (**IV, SFig 1B**). These results strongly imply that Ang2 is involved in the pathogenesis of CNS autoimmune disease.

Ang2 blockade reduces leukocyte infiltration into the SCs

To further analyze the effect of Ang2 on leukocyte recruitment, we enriched immune cells from the SCs of control and Ang2 antibody treated EAE mice. We detected fewer microglia, macrophages, granulocytes, and T cells in the SCs after Ang2 blockade (**IV, Fig. 2A and D**). This also decreased expression of pro-inflammatory cytokines and subunits of α4ß1-integrin, which is involved in T cell trafficking (**IV, Fig. 1B**). In contrast, Ang2 overexpressing mice had higher leukocyte infiltration and increased number of microglia in their SCs (**IV, Fig. 2C and E**). These results indicate that Ang2 regulates leukocyte infiltration in CNS autoimmune disease.

Ang2 regulates immune cell activation

To further analyze the effects of Ang2 on immune cells, we performed single-cell RNA-sequencing (scRNA-seq) of $CD45^+$ cells from the SCs of control, Ang2 antibody-treated and Ang2 overexpressing EAE mice. Among the sorted cells, we identified five main clusters of immune cells, including macrophages, microglia, granulocytes, Th cells, and dendritic cells (**IV, Fig. 3A**). The highest number of significantly downregulated genes was found in the macrophages and microglia. Gene ontology biological process analysis of downregulated genes in microglia and macrophages after Ang2 blockade indicated their involvement in multiple processes. These included antigen processing and presentation, inflammatory response, oxidative stress, regulation of leukocyte cell-cell adhesion, regulation of leukocyte migration, regulation of T cell activation, proliferation, and differentiation (**IV, Fig. 3B and C**). Supporting these findings, Ang2 overexpression increased the expression of genes involved in inflammatory processes (**IV, Fig. 3 B and C**).

Further analysis of the scRNA-seq data suggested that Ang2 modulates the polarization of CNS myeloid cells. Notably, the TREM2-APOE pathway, which drives the transition of microglia from homeostatic to a neurodegenerative/neuroinflammatory phenotype (Krasemann et al., 2017), was downregulated after Ang2 blockade and upregulated in $Ang2^{EC}$ mice (**IV, Fig. 3F**). Similar changes were also detected in MHCII-associated genes, which are associated with a pro-inflammatory phenotype (**IV, Fig. 3F**). These data indicate that Ang2 is involved in the pro-inflammatory polarization of microglia and macrophages.

Ang2 inhibition reduces activation of α5β1-integrin in microglia

To understand how Ang2 modulates polarization of CNS myeloid cells, we analyzed the expression of Ang2 and its receptors Tie2 and α5β1-integrin in microglia and macrophages. In comparison to ECs, we detected low levels of Ang2 and Tie2 but comparable levels of α5β1-integrin in microglia and macrophages (**IV, Fig. 4A and B**). Ang2 has been shown to signal via α5β1-integrin when Tie2 levels are low (Hakanpaa et al., 2015). In line with this, we detected reduced α5β1-integrin activation in the microglia of EAE mice treated with Ang2 antibody (**IV, Fig. 4C**), indicating that Ang2 could regulate microglial polarization via α5β1-integrin.

Ang2 blockade reduces EC inflammation

To understand the mechanisms of Ang2 regulated leukocyte infiltration, we performed scRNA-seq analysis of $CD31^+$ ECs sorted from the SCs of control, Ang2 antibody-treated, and Ang2

overexpressing EAE mice. We identified four main clusters, including venous, capillary-venous, capillary-arterial, and arterial clusters (**IV, Fig. 5A-C**). The capillary-venous ECs showed the highest number of differentially expressed genes, and endogenous Ang2 was most highly expressed in this cluster (**IV, Fig. 5C**). Gene ontology biological process analysis of genes that were significantly downregulated in the capillary-venous ECs of Ang2 antibody-treated mice and upregulated in *Ang2*^{EC} mice indicated their involvement in leukocyte cell-cell adhesion, antigen processing and presentation, response to cytokine, and T cell activation (**IV, Fig. 5D**). Furthermore, Ang2 regulated the expression of EC adhesion molecules, which are known to facilitate leukocyte influx into the CNS in EAE (**IV, Fig. 5G-I**).

Ang2 inhibition reduces inflammation-induced BBB permeability

To investigate the effect of Ang2 on BBB integrity, we analysed leakage of Evans blue into the SCs of EAE mice. Ang2 blockade reduced BBB leakage, whereas Ang2 overexpression increased it (**IV, Fig. 6**). These results are in line with a study showing that Ang2 regulates BBB permeability in a stoke model (Gurnik et al., 2016). Overall, these findings indicate that Ang2 blockade may reduce leukocyte infiltration into the CNS parenchyma via downregulating EC adhesion molecules and reducing BBB leakage.

Tie2 activation by Ang2-binding and Tie2-activating antibody reduces autoimmune neuroinflammation

To find out the possible mechanism of how Ang2 may regulate ECs in neuroinflammation, we treated the EAE mice with ABTAA, which is known to cluster Ang2 for efficient Tie2 activation. Several studies have indicated that Tie2 activation by ABTAA is more beneficial than conventional Ang2 blockade (Han et al., 2016; Park et al., 2016). In line with this, we found that Tie2 activation by ABTAA ameliorates EAE severity (**IV, Fig. 6A and B**), suggesting that converting Ang2 into a Tie2 agonist is beneficial in neuroinflammation. This is supported by previous studies, in which treatment with the Tie2 agonist Ang1 reduced EAE development (Jiang et al., 2014). These data are consistent with a model that Ang2 suppresses Tie2 activation in ECs in neuroinflammation and that enhancing Tie2 phosphorylation has therapeutic potential for the treatment CNS autoimmune diseases.

Ang2 in autoimmune neuroinflammation

In summary, our results indicate that Ang2 is involved in neuroinflammation, and its blockade attenuates leukocyte infiltration into the CNS by downregulating the expression of EC adhesion molecules and by promoting BBB integrity. In addition, Ang2 inhibition reduced the proinflammatory polarization of CNS myeloid cells.

Disruption of the BBB is an early feature of the pathogenesis of MS that contributes to the recruitment of leukocytes into the CNS. Natalizumab, a monoclonal antibody, reduces the number of lesions and clinical relapses in MS by targeting α4β1-integrin, which is required for leukocyte infiltration to the CNS (Polman et al., 2006). This indicates that inhibition of leukocyte recruitment is beneficial in treating MS. However, since α4β1 is expressed in many immune cells, its targeting affects adversely the physiological functions of the immune system and can result in severe side

effects (Langer-Gould et al., 2005). Thus, new treatment strategies that can improve BBB integrity and inhibit leukocyte recruitment into the CNS are needed.

Ang2 blockade seems to attenuate EAE by two main mechanisms: reducing leukocyte infiltration to the CNS and reducing the proinflammatory polarization of CNS myeloid cells. Mechanistically, Ang2 inhibition could regulate microglia polarization via α5β1-integrin. Similar findings were observed in a mouse model of myocardial ischemia, wherein Ang2 regulated macrophage pro-inflammatory polarization via α5β1-integrin in an autocrine manner (Lee et al., 2018). Previous studies have also shown that Ang2 can activate neutrophils and macrophages in a paracrine manner (Lemieux et al., 2005; Scholz et al., 2011; Sinnathamby et al., 2015). Our results indicated that CNS myeloid cells express lower levels of Ang2 than ECs. Moreover, EC-specific overexpression of Ang2 increased EAE disease severity, whilst ABTAA-induced Tie2 activation in ECs reduced EAE clinical symptoms. These results suggest that EC-derived Ang2 is the main regulator of neuroinflammation. However, to determine the exact contribution of EC-derived vs. CNS myeloid-cell derived Ang2 in neuroinflammation, additional conditional cell-type specific Ang2-knockout mouse lines should be investigated. Nevertheless, our results indicate that Ang2 blockade may provide a therapeutic option for the treatment of CNS autoimmune disease.

CONCLUSIONS AND FUTURE PROSPECTS

The Ang-Tie pathway is crucial in vascular development and function. It is also a therapeutic target and thus it is essential to improve our knowledge of this signaling system. In this thesis, we investigated Tie1 and Ang2 in various mouse and cell models to gain new insights into the function and regulation of Ang-Tie signaling. We discovered several previously unknown functions for Tie1 during vascular development and pathological conditions. We found that Tie1 regulates embryonic lymphatic vessel development and postnatal sprouting angiogenesis (I, II). We also discovered that Tie1 regulates tumor angiogenesis and growth (II) and that Ang2 is involved in autoimmune neuroinflammation (IV). Furthermore, we elucidated the signaling mechanisms of Tie1 in Ang-Tie2 interplay and investigated the role of Tie1 in regulation of angiopoietin function (III).

Our work indicates that Tie1 acts as a positive regulator of Tie2 signaling, while other studies have suggested that in certain conditions Tie1 inhibits Tie2 activation. Thus, several unanswered questions remain to be elucidated. Based on our results, Tie1 and Tie2 can directly interact, and this interaction regulates the activities of Ang1 and Ang2 on Tie2 (III). The consequence of this interaction is likely further regulated by the local cellular milieu, especially by the relative ratio of Ang1 and Ang2, which dynamically changes in various physiological and pathological conditions. In line with this, Savant et al. proposed a model wherein Tie1 differentially regulates Tie2 in tip vs. stalk cells during retinal sprouting angiogenesis (Savant et al., 2015). Tie2 is also differently regulated at cell-cell junctions versus cell-matrix contacts (Fukuhara et al., 2008; Saharinen et al., 2008). Thus, different localization of the receptors might explain the differences observed in Tie1 functions. Future studies utilizing for example optogenetically spatially controlled Tie1 and/or Tie2 deletion would facilitate our understanding of this topic.

Another example of context-dependency of Tie1 function is that Tie1 deletion in embryos induced hemorrhage and loss of microvascular integrity (Puri et al., 1995), whereas Tie1 deletion in tumors prevented tumor growth (II), vessel leakage and increased Tie2 activation (La Porta et al., 2018). Additionally, we found that inflammation-induced Tie1 cleavage is associated with increased vascular permeability and reduced Tie2 activation (III). These data suggest that Tie1 has different roles during vascular development vs. pathological angiogenesis vs. acute inflammation. Thus, additional studies are needed to fully understand the complex role of Tie1 in Tie2 signaling and to determine the mechanisms regulating its function in different conditions. For example, to dissect the role of Tie1 in acute vs. chronic inflammation, mouse studies utilizing cleavage-resistant Tie1 or cleavage inhibition would be highly beneficial.

Although most studies focus on the functions of Tie1 in relation to Tie2 signaling, Tie1 has also Tie2-independent functions. For example, Tie1 and Ang2 are both required for postnatal lymphatic development in the ear skin, while Tie2 is dispensable (Dellinger et al., 2008; Shen et al., 2014a). In addition, Tie1 and Ang2 regulate sprouting angiogenesis in the retina and in tumors (II), whereas Tie2 is not expressed in the tip cells of angiogenic vessel sprouts (Felcht et al., 2012). Previous studies have indicated that Ang2 acts via α5β1-integrin in cells where Tie2 levels are low (Felcht et al., 2012; Hakanpaa et al., 2015). Additionally, we showed that α5β1-integrin was required for Ang1-induced Tie1 phosphorylation (III) and others have shown Tie1 to associate with integrins (Dalton et al., 2016). Future studies investigating the interactions of Tie1 and the integrins might shed light to the mechanisms of Tie2-independent functions of Tie1. Furthermore,

analysis of the recently found Tie1 ligand, LECT2 (Xu et al., 2019), could provide new insights into Tie1 signaling.

Limited knowledge of the signaling mechanisms of the Ang-Tie system has complicated the development of drugs targeting this pathway. Treatments targeting the Ang-Tie pathway have been in development for neovascular eye diseases and cancer. Furthermore, targeting the Ang-Tie pathway have shown promising results in preclinical models where vascular integrity is compromised such as sepsis. Unfortunately, development of Ang2 targeting antibodies for cancer treatment (MEDI3617), was interrupted in clinical trials due to insufficient efficacy (Hyman et al., 2018). Thus, new treatment strategies are needed.

In study II, we demonstrated that Tie1 was required for retinal sprouting angiogenesis as well as for tumor angiogenesis and growth. Furthermore, our results indicate that inflammation induced Tie1 cleavage contributes to loss of Ang2 agonistic activity (III). Thus, our studies suggest that Tie1 is a potential therapeutic target in angiogenesis-related disorders and in inflammation. Our results also suggest that Tie1 could be used as a biomarker in human inflammatory diseases (III) and, in line with this, recent data has shown a prognostic role for Tie1 levels in metastatic breast cancer (Tiainen et al., 2019). Current anti-angiogenic therapies have focused mainly on targeting the VEGF/VEFGR pathway, which has resulted in limited success. Thus, new anti-angiogenic therapies are being developed for combinatorial targeting with VEGF/VEFGR blockers. Our data from tumor isografts in mice indicates that Tie1 deletion has synergistic inhibitory effects on tumor growth in combination with angiopoietin therapy (II). Additional studies are needed to validate the targeting of Tie1 in combination with VEGF or Ang2 blockers or in combination with cancer immunotherapies.

In study IV, we investigated the function of Ang2 in EAE, which is the most common mouse model of MS. We demonstrated that Ang2 is involved in EAE pathogenesis via its effects on both ECs and immune cells. Ang2 blockade reduced leukocyte infiltration into the CNS. In this regard, the clinically available Ang2-blocking antibodies could provide an alternative therapeutic option for the treatment of MS. Previous study has implicated Ang2 in stroke (Gurnik et al., 2016), yet further studies are required to ascertain if Ang2 is involved in other neurological disorders such as Alzheimer's disease. Similar to Ang2 blockade, Tie1 deletion is shown to reduce the expression of EC adhesion molecules (II, III, IV (Woo et al., 2011)). Indeed, it would be interesting to investigate whether Tie1 targeting might result in beneficial effects in neuroinflammation.

Overall, this thesis provides new information on the complex functions of the Ang-Tie pathway. Of clinical relevance, our work reveals the involvement of Tie1 in tumor angiogenesis and in inflammation as well as the function of Ang2 in neuroinflammation. We also found that Tie1 has a role in lymphatic vessel development. In the future, better understanding of the Ang-Tie pathway should help in the development of treatment strategies for (lymph)angiogenesis- and inflammation-related diseases.

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